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Attempts to protect cats against FIV infection by prophylactic administration of feline interferon Ω

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DEDICATION

TO THE MEMORY OF

MY PARENTS

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ABBREVIATIONS

- ALAT (GPT) alanine amino transferase
- AP-1 activator protein 1
- AP-4 adaptor protein 4
- ARC AIDS-related complex
- ASAT (GOT) aspartate amino transferase
- ATF activating transcription factor
- CID 50 cat infectious dose 50
- CLCA1 chloride channel calcium activated “family member 1”
- CLMF cytotoxic lymphocyte maturation factor
- Con A concanavalin a
- Ct cycle threshold
- ELISA enzyme-linked immunosorbent assay
- FCoV Feline Corona Virus
- FCV Feline Calici Virus
- FCS fetal calf serum
- FeLV Feline Leukemia Virus
- FHV-1 Feline Herpes Virus
- FIV Feline Immunodeficiency Virus
- FPV Feline Parvovirus
- GAPDH glyceraldehyde-3-phosphate dehydrogenase
- GL 8 Glasgow 8 FIV strain
- HBSS Hank’s balance salt solution
- HBV hepatitis B virus
- HCC hepatocellular cancer
- HCT hematocrit
- IL interleukin
- IL-10 ht2 Interleukin 10 haplotype 2
- LPS lipopolysaccharide
- MCH mean corpuscular hemoglobin
- MCHC mean corpuscular hemoglobin concentration
- MCV mean corpuscular volume
- mRNA messenger ribonucleic acid
- NF1 neurofibromin 1

- NKSF — natural-killer cell stimulatory factor
- OD — optical density
- QTL — quantitative trait locus
- rpm — revolution per minute
- RT — reverse transcriptase
- RT-PCR — reverse transcription polymerase chain reaction
- SH2D1A — Src homology domain protein 1A
- SIDS — sudden infant death syndrome
- TM — transmembrane
- TNA — total nucleic acid
- TSF — t-cell stimulating factor
- Z2 — Zurich 2 FIV strain

ABSTRACT

The prophylactic treatment efficacy against experimental FIV infection of a recombinant feline interferon, (rFeINF Ω) was evaluated with the aim in order to determine whether INFΩ could be used as prophylactic regimen in cats being introduced to a boarding cattery. There seemed to be a chance for success as INF Ω had been found to inhibit FIV in vitro.
The experiment was conducted as a double - blinded study against a placebo.
The efficacy of IFN Ω was evaluated by the following parameters: by the detection of antibodies to TM by ELISA, by clinical chemistry and hematology examinations, by detection and quantitation of proviral and viral loads in blood, plasma and saliva.
IFN Ω - treatment did not lead to changes in the cat health nor did it induce any differences in the course of seroconversion, viral and proviral loads, cytokine expression and weight gain. Therefore, at present use of IFN Ω cannot be considered to have any protective effect in cats against potential FIV infection as it has be expected in a cat catteries with higher risk exposure.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde der Effekt einer prophylaktischen Behandlung von Katzen mit einem rekombinanten Interferon (rFelNF Ω) gegen eine experimentelle Infektion mit FIV untersucht. Mit dieser Arbeit sollte die Frage abgeklärt werden, ob prophylaktische Behandlung min Interferon Ω in der Lage ist, eine Virusinfektion min FIV zu verhindern, respektive in ihrem Verlauf im Vergleich zu nicht behandlten Kontrolltieren zu verzögern und abzuschwächen. Diesem Experiment wurde eine gewisse Chance auf Erfolg zugesprochen, da Interferon Ω in vitro gegen FIV eine deutliche Hemmwirkung gezeigt hatte. Im vorliegenden Experiment wurden 10 Tiere mit Interferon Ω vorbehandelt, 10 Tiere dienten als nicht behandelte Kontrollen. Der Verlauf der anschliessenden Infektion mit einer minimalen Dosis von FIV wurde anhand virologischer (RNA Virusload, DNA Provirusload, Serologie) sowie hämatologischer und klinischer Daten untersucht.

Die Behandlung mit Interferon Ω zeigte keinerlei Effekt auf die Viralen, klinisch-chemischen und hämatologischen Parameter. Deshalb kann INFΩ zur Zeit nicht als wirksam betrachtet warden beim Schutz vor einer FIV-Infektion, wie sie in Katzenheimen vorkommen kann.

1. Introduction and objectives:

1.1. Introduction:

Feline Immunodeficiency Virus (FIV) which was discovered in California in cats with immune suppression is a retrovirus that is transmitted from cat to cat mainly by bites. Related Lentiviruses have been isolated from sheep, horses, cattle, goats, poultry, cats, monkeys, and humans {Desrosiers, el al. 1987}. As all retroviruses FIV contains single stranded RNA and belongs to the genus Lentivirus of the family Retroviridae. Due to structural and biological similarities, FIV can serve in many aspects as model for human immunodeficiency virus (HIV) and AIDS {Steinrigl, et al. 2003}.
In particular, FIV has been extensively used for testing strategies of the development of anti-HIV-1 vaccines, with outcomes that have ranged from complete immunity to absolute lack of protection or even enhanced susceptibility, depending on the types of immunogen and viral challenge used {Boretti et al. 2000, Simone, et al. 2002}.

1.2. FIV and it is important to cats:

FIV severely impairs the immune function of infected domestic cats by destroying the CD4+ lymphocytes {Torten, et al. 1991}.
The prevalence of FIV infection varies greatly among different countries. An overview on published frequencies is given in table 1.

Table 1: prevalences of FIV infection in different countries:

Country	**% (approximately)**	**Ref.**
Belgium	11.3	{Dorny, et al. 2002}
Denmark	6	{Kristensen, et al. 1989}
Germany	2-8.4	{Fuchs, et al. 1994; Hartman and Hinze, 1991; Neu et al. 1998}
Finland	4-7	{Sukura, et al. 1992}
France	22	{Lutz, et al. 1988; Moraillon, 1990}
Gross Britain	6[a] – 19[b]	{Bennett, et al. 1992; Gruffydd-Jones, et al. 1988; Hosie, et al. 1989}
Italy	12.5 – 24[b]	{Bandecchi, et al. 1992; Peri, et al. 1994}
Norway	5.9[a] – 10.1[b]	{Ueland and Lutz, 1992}
Switzerland	0.7[a] – 3.4[b] 1.7 [a] – 3.0[b]	{Lutz, et al. 1988; Lutz, et al. 1990} {Gruber, 2000}
Spain	8.3[a] – 13.9[b]	{Arjona, et al. 2000}
Turkey	22.3	{Yilmaz, et al. 2000}
USA	3.6[a] - 15 [b] 1.2 – 14 5.2	{Grindem, et al. 1989} {Yamamoto, et al. 1989} {Luria, et al. 2004)
Australia	13.5 – 32 6.5[a] – 20.8[b] 4.9[a] – 16.7[b]	{Belford, et al. 1989; Sabine, et al. 1988} {Malik, et al. 1997} {Muriden, 2002}
Newsland	37	(Swinney, et al. 1989}
Japan	12.4 – 43.9 9.8 - 24	{Furuya, et al. 1990; Ishida, et al. 1989} {Maruyama, et al. 1988; Maruyama, et al. 2003}

[a] healthy cats

[b] sick cats

1.3. Objectives:

Cats are susceptible to several viral infections which are mainly transmitted by direct contact with other cats. In this context boarding catteries or shelters are especially harmful as in these environments risks from a wide range of origins are accumulated.
IFN Ω was shown to have anti- viral effects in vitro against FIV and FPV and probably other viruses as well {Tanabe, et al. 2001}. It was the goal of this project to evaluate in a double-blind study the effect of IFN Ω to protect at least to some degree cats from subsequent experimental FIV infection. If this treatment was successful IFN Ω could be used also under field conditions to protect cats from FIV and possibly other infections for at least a short period. This could be the basis for a standard treatment of cats that are to be held at a boarding cattery or a shelter for a short time.

2. Literature:

2.1. FIV, General:

Various publications concerning FIV have been discussed in the dissertations of Kim Bauer {1994}, Christian Leutenegger {1995}, Felicitas Boretti {1999} and Caroline Mislin {2002}. Besides important general aspects of FIV discussed in the early years after discovery of the virus, this work will mainly focus on the more recent studies aiding further characterization and understanding of FIV infection.

2.1.1. Viral Morphology and Genomic Structure:

First discovered in California by Pedersen and colleagues in 1987, FIV is a typical lentivirus belonging to the family retroviridae. Mature viral particles are spherical to ellipsoid, measure slightly over 100 nm in diameter, and present few, short spikes on the outer side of their envelope {Miyazawa, 1989}. FIV virions are diploid, containing two copies of positive sense RNA. Of approximately 10 kB in length, the FIV genome presents several typical characteristics of the retrovirus family. It contains from 5' to 3' three genes, *gag*, *pol*, *env*, that encode for proteins essential to viral structure and replication {Talbott, et al. 1989}:

gag (group specific antigen): encodes for the viral matrix (MA), capsid (CA) and nucleocapsid (NC) proteins

pol (polymerase): encodes for enzymes, including reverse transcriptase (RT), protease (PR), integrase (IN), and dUTPase

env (envelope): encodes for retroviral envelope proteins designated as surface glycoprotein (SU) and transmembrane polyprotein (TM).

The genome is flanked at each end by repeated sequences called long terminal repeats (LTR), comprised of untranslated 3' (U3, promoter/enhancer elements), repeat (R) and untranslated 5' (U5) regions. General functions of LTR include insertion of the viral genetic information in the host's cellular genome and enhancement of its transcription. Moreover, mutational analyzes have revealed numerous elements in the LTR U3 region such as AP-1, AP-4, ATF and NF1 binding sites, which constitute promoter sequences influencing viral gene expression {Sparger, et al. 1992}. Recent studies have also described elements both within U5 and the 5' end of *gag,* which are required for efficient packaging of newly produced FIV, thus allowing expansion of infection in the host {Kemler, et al. 2004}.

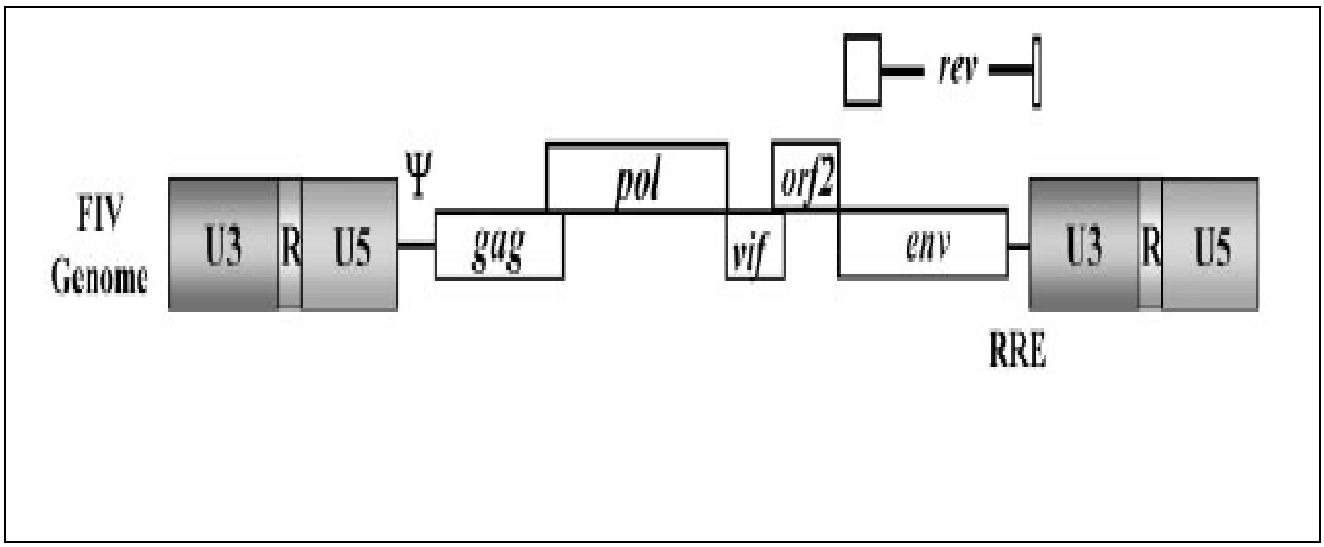


Figure 1 schematic representation of the FIV integrated provirus

Other important regulatory genes of the FIV genome, shared with all or only certain members of the retrovirus family, include a *rev* gene (regulator of virion protein expression), a *vif* gene (viral infectivity factor), and an *Orf-2* gene (also designated *Orf-A*). *Rev* is vital for replication and packaging of all lentiviruses. Its translated protein acts as transporter of viral mRNA from nuclear localization to the cytoplasm, after interaction with a viral factor, the Rev-responsive element (RRE) {Tomonaga, et al. 1994}. Most known lentiviruses, include a *vif* gene, coding a viral infectivity factor responsible for production of infectious viral particles in producer cells and spread of infection to new target cells or tissues {Lockridge, et al. 2000}. FIV transactivation was earlier thought to be dependent on *Orf-2*, a short fragment placed within the vif sequence, with characteristics similar to those of the *tat* (transactivator) gene of the ungulate lentiviruses {de Parseval, et al. 1999}. Interestingly, unlike other lentiviral transactivators, FIV Orf-2 was reported to require additional LTR elements for transactivation {Chatterji, et al. 2002}. Moreover, recent studies indicated that Orf-2 function involves multiple steps of the FIV life cycle including both virion formation and infectivity {Gemeniano, et al. 2004}. The possibility that *Orf-2* function resembles the *vpr* accessory genes of other lentiviruses rather than *tat,* is currently discussed.

2.1.2. Viral Life Cycle:

2.1.2.1 Receptor Usage and Cell Entry:

FIV replication is similar to that of other retroviruses. The first essential step for effective infection of the target cell is the attachment of the virion to the cell surface. For efficient binding, viral surface glycoproteins and receptors of the outer cellular membrane both play an important role. Unlike HIV, the CD4 molecule is not used by the feline virus as primary

receptor or co-receptor {Hosie, et al. 1993}. CD9 was earlier thought to be a main receptor for FIV, but further studies have demonstrated that antibodies directed against CD9 block virus release rather than viral entry {de Parseval, et al. 1997}.

With reference to knowledge on receptor usage by HIV, importance of chemokine receptors as means of target cell entry by FIV were more deeply studied. FIV was soon shown to utilize CXCR4 as a receptor for env-mediated fusion, and both primary as well and laboratory adapted strains proved to need the receptor for host cell infection {Richardson, et al. 1999}. Further studies had identified the second extracellular loop of CXCR4 as the primary determinant of target cell infection by FIV {Willett, et al. 1998}. Moreover, in vitro FIV infection was shown to be inhibited by two different CXCR4 ligands, stromal cell derived factor 1 alpha (SDF1α) {Endo, et al. 2000} and the bicyclam, AMD3100 {Egberink, et al. 1999}. However, some CXCR4 positive cell lines were resistant to FIV infection indicating the probable role of an additional receptor. The use of other chemokine receptors, such as CCR5 and CCR3 has also been demonstrated, as antibodies against CCR3 as well as RANTES, a ligand to CCR5, were able to inhibit FIV infection of target cells {Lerner, et al. 2000}.

Recently, CD134, a T-cell antigen and co-stimulatory molecule was recognized as primary receptor for FIV {Shimojima, et al. 2004}. CD 134 expression was demonstrated to promote viral binding and render cells permissive for viral entry, but productive infection remained CXCR4 dependent. The tropism of FIV in vivo seems to be consistent with the predicted expression of CD 134. In agreement with this is the observation that health and survival of FIV-infected cats was better in cats that developed autoantibodies to CD134, the major receptor of FIV {Grant, et al. 2009}

This can readily be explained by the fact that these antibodies compete with FIV for attachment to the receptor.Viral characteristics critical for fusion with target cells appear to rely on a limited number of regions of the surface and transmembrane glycoproteins. The V3 loop of gp 120 viral surface glycoprotein has been shown to be critical for viral binding {Verschoor, et al. 1995}, and the V3-V5 region mediates chemokine receptor use {Johnston, 2002}. Additionally, a tryptophan-rich motif present membrane-proximally in the ectodomain of the FIV transmembrane glycoprotein, proved to be essential in the processes of fusion and viral entry {Giannecchini, et al. 2004}. To get more insight into the role of APOBEC3 (A3) cytidine deaminases in the species-specific restriction of feline immunodeficiency virus (FIV) of the domestic cat, Jörg et al. {2010} tested the A3 proteins present in big cats (puma, lion, tiger, and lynx). These A3 proteins were analyzed for expression and sensitivity to the Vif protein of FIV. While A3Z3s and A3Z2-Z3s inhibited Δ*vif* FIV, felid A3Z2s did not show any antiviral activity against Δ*vif* FIV or wild-type (wt)

FIV. All felid A3Z3s and A3Z2-Z3s were sensitive to Vif of the domestic cat FIV. Vif also induced depletion of felid A3Z2s. Tiger A3s showed a moderate degree of resistance against the Vif-mediated counter defense. These findings may imply that the A3 restriction system does not play a major role to prevent domestic cat FIV transmission to other *Felidae*. In contrast to the sensitive felid A3s, many nonfelid A3s actively restricted wt FIV replication. To test whether Vif_{FIV} can protect also the distantly related human immunodeficiency virus type 1 (HIV-1), a chimeric HIV-1.Vif_{FIV} was constructed. This HIV-1.Vif_{FIV} was replication competent in nonpermissive feline cells expressing human CD4/CCR5 that did not support the replication of wt HIV-1. We conclude that the replication of HIV-1 in some feline cells is inhibited only by feline A3 restriction factors and the absence of the appropriate receptor or coreceptor. {Jörg, et al. 2010}.

2.1.2.2. Cellular Tropism:

The receptor of the target cell surface determines the ability of FIV to gain entry into the cell and to establish productive infection. In vivo, FIV replicates in CD4+ and CD8+ lymphocytes {Brown, et al. 1991}, in B lymphocytes {Dean, et al. 1996}, in macrophages (Brunner, et al. 1989), monocytes {Dow, et al. 1999} as well as in astrocytes and microglia {Dow, et al. 1990}. Some strains replicate preferentially in lymphoctytes (lymphocytotropic strains) and only minimally in monocytes, while other strains are able to replicate equally well in both cell types (monocytotropic strains). Moreover, certain strains have been shown to replicate in Kupffer cells {Martin, et al. 1995}, suggesting that these cells may play a role in the pathophysiology of FIV infection. In vitro, the cellular tropism of primary isolates is restricted to mitogen-activated PBMC, dendritic cells, macrophages and thymocytes {Lerner, et al. 1998}. Only one T-lymphoblastoid cell line, obtained from a specific pathogen free (spf) cat and characterized as Pan T+, CD4-, CD8-, was described to show a cytopathic effect upon inoculation with FIV {Matteucci, et al. 1995}. Adapted strains can replicate in fibroblastoid cell lines, the most commonly used being Crandell-Rees Feline Kidney (CRFK) cells. Interestingly, a human lymphoblastoid cell line {Ikeda, et al. 1996} as well as human PBMC {Johnston, et al. 1999} have efficiently been infected with FIV, but failure in the transcription step of replication caused latency of the virus in these cells. Infection of human cells by FIV was the first evidence of shared chemokine receptor use between primate and non-primate lentiviruses and represented a relevant step for better understanding of the AIDS pathogenesis {Willett, et al. 1997}.

2.1.2.3. Replication and virus assembly:

After fusion of external viral and cellular proteins and FIV penetration of the cell, the viral core is freed in the cytoplasm, undergoing simultaneously specific structural changes. The retroviral RNA within the modified core is reverse transcribed by the viral reverse

transcriptase (RT) first to a linear single-stranded, later to a double-stranded DNA with long terminal repeats (LTR). The newly produced retroviral DNA is still associated with some viral enzymes and core proteins; together they form the preintegration complex. FIV posseses the capacity to infect both dividing and non-dividing cells. In dividing cells, the preintegration complex can access the host DNA easily during mitosis. For infection of non-replicating cells, active transport of the preintegration complex in the cellular nucleus is necessary. A polypurine tract located centrally in the FIV genome and designated as central DNA flap has been held responsible for this function {Whitwam, et al. 2001}. The viral integrase (IN) catalyzes the integration of viral DNA in the host's cell DNA. Interestingly, the integration of the virus does not take place at a specific site in the host genome, but rather occurs randomly at different sites.

Integrated viral DNA, called provirus, behaves very much like a eukaryotic gene. It may be transcribed into full-length transcripts using host cell RNA Polymerase II in order to produce more virus, or it may remain latent for long periods of time and replicate when cellular DNA is replicated by the cell. Each infected cell thus transmits the viral genome to its descendants.

For the efficient production of new virus particles, the full-length transcripts follow three different routes. Some are exported from the nucleus and serve as mRNA for viral *gag* and *pol* protein precursors, which are translated by cytoplasmic ribosomes. Other full-length RNA molecules are spliced directly in the nucleus to form mRNA for the env protein precursors. Env mRNA is translated by ribosomes bound to the endoplasmatic reticulum, the synthesized proteins are glycosylated in the golgi apparatus and cleaved by cellular proteinases to form the mature TM-SU complex. These mature proteins are then delivered to the surface of the cell. Finally, other full-length transcripts are transported into the cytoplasm and incorporated into the budding viral particles where they serve as progeny viral genomes.

All viral parts produced in this way assemble at budding sites at the surface of the plasma membrane. During budding, the virus receives its envelope, consisting of parts of the cell membrane and viral glycoproteins. Simultaneously, the viral encoded protease, which is itself a component of the core precursor protein, cleaves at specific sites within *gag* and *pol* precursors to produce mature proteins that allow assembly and release of infectious viral particles, able to infect new target cells in the host. The main steps of retroviral life cycle are summarized in figure 2.

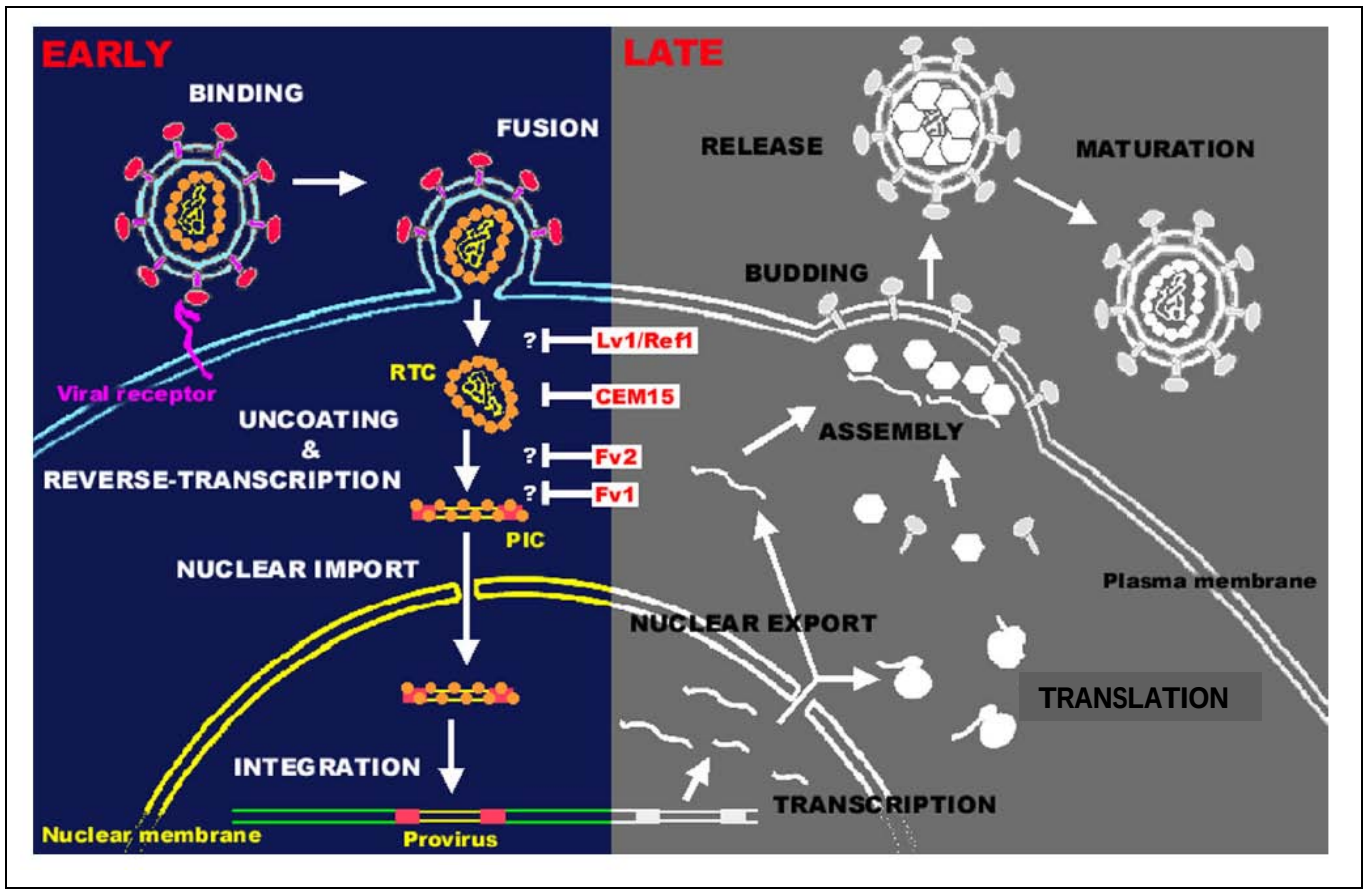


Figure 2:- A schematic view of early and late stages of the retroviral replication cycle

2.2. Pathogenesis:

2.2.1. Viral Transmission:

Virus can be isolated by cell or tissue culture {Yamamoto, et al. 1988}; {Dow, et al. 1990}. Under field conditions, shedding in saliva is most relevant, and aggressive biting is considered the most important route of transmission. Indeed, older, free-roaming male cats are more prone to infection due to pronounced territorial behaviour. In addition, cats living in environments with high population density belong to high-risk groups, although the virus is not efficiently spread by casual non-aggressive contact between cats. When kept strictly indoors, cats rarely become infected and a low prevalence in breeding cats is predominantly due to the fact that they have no chance to become infected.

FIV is present in semen of naturally and experimentally infected cats {Jordan, H. et al. 1998}, and veneral transmission from infected males to non-infected females is possible. Artificial insemination performed with fresh semen from experimentally infected cats effectively infected FIV-naïve queens in 50% of the cases {Jordan, et al. 1996}. Admittedly, mating in cats is often accompanied by biting, and the relevance of mating as a route of transmission under field conditions seems very low.

Of particular interest is the possibility of vertical transmission resulting in productive infection and disease in offspring. Trans-placental transmission of FIV to the kittens occurred in 2/3 cases after either intravenous or subcutaneous infection of the queen three weeks prior to parturition {Wasmoen, et al. 1992}. Moreover, mothers inoculated with FIV immediately postpartum can infect newborn kittens via milk {Sellon, et al. 1994}.
Interestingly, more recent studies show that the frequency of perinatal FIV transmission from infected queens to their kittens seems to correlate with chronicity of infection, and maternal symptoms of clinical immunodeficiency {O'Neil et al. 1996}.
In experimental studies, transmission via mucosal and parenteral (intravenous, intraperitoneal, subcutan) routes have proven to be efficient. Virus strains representing at least three clades of FIV can be transmitted across the vaginal, rectal, or oral mucosa {Obert, et al. 2000}. However, up to 10e4 fold more viruses are required for infection by mucosal routes in comparison to parenteral routes {Bishop, et al. 1996}.

2.2.2. Course of Disease:

Kinetics of FIV infection varies depending on virus isolate and route of exposure. The following refers to a general pattern of infection, which can be approximately extrapolated to the various forms of FIV disease.
The course of FIV infection is divided into five clinically recognizable phases, which naturally correlate with pathogenetic progression of disease: 1) acute infection, 2) asymptomatic phase, 3) persistent generalized lymphadenopathy, 4) ARC, and 5) AIDS phase {Ishida, et al. 1990}.
The clinical aspects of theses stages are described in more detail under 2.4.
FIV infection is characterized by progressive immune deterioration. Hallmarks of disease progression include decreasing CD4+ lymphocyte counts in blood and CD4+:CD8+ lymphocyte ratio {Callana, et al. 1992}, as well as proviral load in peripheral blood mononuclear cells (PBMC) {Hohdatsu, et al. 2005} and viral load in plasma {Diehl, et al. 1996}.
Lymphoctyes of CD4+ phenotype are primary targets of infection. Additionally, a significant infection of macrophages takes place already during the acute phase of the disease. Proviral DNA can thus be deteced in PBMC as early as five days after infection, and infectious virus can be isolated from lymphocytes as of day 10 post infection.Viremia then rapidly increases until week 3, peaks between weeks 7 and 8 and then decreases again during the asymptomatic phase of disease. In the terminal stage, virus replicates again efficiently and viral load increases accordingly in plasma. When virus peaks in the acute phase of infection, CD4+ cells decrease by approximately one third due to primary viral

replication in these cells. However, a slow rise can be observed as viremia severity decreases. During the asymptomatic phase, CD4+ cells decrease only very slowly, while a very rapid decrease occurs during the terminal AIDS stage of disease. As early as two weeks after the onset of infection, the CD8+ lymphocyte population expands rapidly. The antiviral activity of this population subset implicates reduction of viremia. During the asymptomatic phase of disease, the total CD8+ lymphocyte count is stabilized at a rate higher than normal level and decreases again to very low counts in terminal stages of disease.

Antibodies against FIV have been detected in experimentally and naturally infected cats as early as 2-3 weeks following infection and persist throughout infection. High antibody levels correlate with the viremia peak in the acute phase of infection.

2.2.3. Immunological Aspects:

The pathogenesis of FIV is to date not completely understood. Infected cats develop latency despite elaboration of defence mechanisms such as specific cellular immune responses and production of neutralizing antibodies.

This chapter represents an overview of the important known immunological processes during FIV infection of the domestic cat.

2.2.3.1. CD4+ T-Lymphocyte Depletion:

All FIV isolates and all routes of infection commonly lead to a decline of CD4+ lymphocyte count in the peripheral blood of the infected cat. AIDS stage of disease is often defined by a concentration of less than 200 CD4+T-cells/µl whole blood, although CD4+ cytopenia is clearly not responsible alone for terminal stage immune dysfunction.

Decrease in CD4+ cells depends on several mechanisms but is usually due to a reduced life span of the cells. The quantitative decrease, however, cannot just be explained by cytolysis as a result of viral infection, because the percentage of infected cells is significantly lower than the number of cells dying. As a consequence, FIV-mediated T cell depletion and lymphocyte activation have been related to an accelerated cell death or apoptosis and it has been shown that lymphocytes from FIV-infected cats are prone to die after short-term culture in vitro {Guiot, et al. 1997}. Additionally, the extent of in vitro lymphocyte apoptosis was shown to correlate with progression of disease {Holznagel, et al. 1998}.

More recent and detailed information on T cell depletion and apoptosis is discussed under 2.2.3.3.

2.2.3.2. CD8+ T-Lymphocyte Anti-Viral Activity:

Rapid expansion of the CD8+ lymphocyte subset along with specific anti-FIV cytolytic and non-cytolytic mechanisms arises early in the course of FIV infection, even before a

noticeable humoral immune response. Appearance of this antiviral activity corresponds to a reduction in viremia and transition to the asymptomatic stage of disease {Willett, et al. 1993}. Interestingly, although cytotoxic T-lymphocyte (CTL) responses against gag and env antigens have been described, persistent high-level circulating antiviral CTL could not be detected during FIV infection, as has been observed in human immunodeficiency virus-infected humans {Beatty, et al. 1996}. CTL responses seem to become localised in the lymph nodes and spleen with progression of disease {Flynn, 2002}. Detectable earlier than cytotoxic mechanisms, non-cytolytic processes have a more diverse action potential. The inhibitory effect is mediated by soluble factors and as a consequence, not dependent on direct cell-to-cell contact {Hohdatsu, et al. 1998}. Further studies allowed to more precisely allot this CD8+ T cell anti-FIV activity at the level of FIV mRNA synthesis from the FIV proviral DNA {Hohdatsu, et al. 2000}. Moreover, acute stage of FIV infection is characterized by the appearance of a CD8+ T cell subpopulation showing reduced expression of the CD8 β chain and complete disappearance of the L-selectin CD62L surface molecule {Gebhard, et al. 1999}.

These CD8βlowCD62L T cells then persist throughout the course of infection, and recent studies have demonstrated that the CD8+ T cells, which specifically respond to FIV antigens in the course of infection, are contained in this T cell subpopulation {Paillot, et al. 2005}**.**

FIV replication is controlled to a significant degree by IFN-γ produced in CD8+ cells. It was recently shown that CD4 (+) CD25 (+) Treg cells have a negative effect on the CD8(+) immune response during the acute and chronic stages of FIV infection {Folge, et al. 2010}.

2.2.3.3. T Cell Dysfunction, Anergy and Apoptosis:

Already in the acute phase of infection, defects in T cell responses are encountered. Mitogen and antigen induced IL-2 production and lymphoproliferation are reduced in the acute phase and continue to decline with progression of disease {Torten, et al. 1991}. The B7.1 and B7.2 costimulatory molecules on antigen-presenting cells provide second signals for regulating T cell immune responses via CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4) on T cells. CD28 is a marker of cell proliferation, whereas CTLA4 is a marker of anergy or apoptosis, terminating the immune response. Flow cytometry revealed high percentages of CD8+ and CD4+ cells expressing B7.1, B7.2, and CTLA4 in lymph nodes of FIV-NCSU1-positive cats and a large fraction of CTLA4+ T cells coexpressing B7.1 and B7.2 {Tompkins, et al. 2002}. Moreover, anti-B7.1 antibodies significantly inhibited T cell apoptosis in FIV-infected cats with low-level plasma viremia, further suggesting that lymph node apoptosis and immune deterioration in FIV-infected cats may

result from chronic B7-CTLA4-mediated T-T negative signalling interactions {Bull, et al. 2004}.

Further understanding of mechanisms for T-cell depletion and dysfunction comes from studies of regulatory T-cells. CD4+ regulatory cells prevent the activation of autoreactive T cells, and help to maintain self-tolerance. Detection of CD25 (the IL-2 receptor alpha chain) has been used to identify the subpopulation of CD4+ T cells which have regulatory function. CD25+ subsets of CD4+ as well as CD8+ T cells increase in lymph node of FIV-NCSU1 infected cats very early after exposure to virus. The CD4+CTLA4+B7+ phenotype described in FIV positive cats interestingly resembles the CD4+CD25+CTLA4+ phenotype described for immunosuppressive T regulatory (Treg) cells. Additionally, similar to Treg cells, feline CD4+CD25+ T cells directly isolated from LN of FIV infected cats do not produce IL-2, fail to proliferate in response to mitogen stimulation, and suppress the proliferative response and the IL-2 production of Con A-stimulated autologous CD4+CD25- T cells. These FIV negative, activated, anergic, immunosuppressive CD25+CTLA4+B7+CD4+ Treg-like cells may contribute to the progressive loss of T cell immune function that is characteristic of FIV infection {Vahlenkamp, et al. 2004}.

Further characterization of these T cell subpopulations indicate possible mechanisms of FIV latency, typically encountered during FIV infection. While both CD4+CD25+ and CD4+CD25- cells are susceptible to FIV infection in vitro and in vivo, only CD4+CD25+ cells produce infectious virions when cultured with IL-2. Latently infected CD4+CD25- cells produce infectious virions only following (ConA) stimulation. Furthermore, CD4+CD25+ cells remain relatively resistant to apoptosis, whether or not infected with FIV. Together, all these findings define CD4+CD25+ cells as a main sources of productive FIV infection, whereas CD4+CD25- cells represent a potential latent viral reservoir capable of being reactivated after stimulation {Joshi, et al. 2004}.

2.2.3.4. Cytokine Production:

Dysregulation in cytokine expression has been well described in acute and chronic FIV infection. In FIV infected cats, CD4+ lymphocytes produce TNF-α, IFN-γ, IL-2, IL-4, and IL-10, while CD8+ lymphocytes express TNF-α, IFN-γ and IL-2. Monocytes and macrophages are the source of IL-1, IL-6, TNF-α, IL-10 and IL-12p40 {Burkhard, et al. 2003}. Whole blood and relevant lymphoid tissues such as lymph nodes, spleen and thymus demonstrate unique cytokine profiles that differ qualitatively and quantitatively during infection, correlating with virus replication in each tissue or organ {Dean, et al. 1998}.

Serum levels of IL-1, IL-6, TNF-α and IFN-γ are increased with FIV infection and are higher in symptomatic versus asymptomatic cats {Lawrence, et al. 1995}.

It is well established that cytokines such as IFN-γ and TNF-α produced by T cells upon antigen stimulation are important for controlling viral infections. Increasing TNF-α level during the acute phase of infection is associated with virus replication {Kraus, et al. 1996}. It has now also been demonstrated, that FIV infected cats develop both IFN-γ + and TNF-α + FIV specific T cell subpopulations early in the course of infection {Paillot, et al. 2005}. IFN-γ, also called type II IFN, is produced by activated T cells and natural killer (NK) cells and is known for its immunomodulatory and antiviral activities. Surprisingly, in contrast to feline type I IFN, which have been reported to inhibit *in vitro* replication of FIV as well as other common feline viruses {Yamamoto et al. 1990}, feline INF-γ seemingly lacks inhibiting potential of infection of PBMC {Tanabe, et al. 2001}. These results suggest that the main function of IFN-γ produced during infection may not be the direct inhibition of FIV replication. Instead, indirect effects of IFN-γ such as increase of cytotoxic T cell and NK cell responses to virus-infected cells may play a more central role in controlling the infection. Nevertheless, it is accepted that presence of IFN-γ production during FIV infection reflects a functional immune system, whereas the loss of IFN-γ production correlates with progression to AIDS.

TNF-α production plays an important role in the pathogenesis of FIV. Production of this cytokine correlates with viral replication in serum and tissues {Kraus, et al. 1996}. Moreover, TNF-α is apparently associated with an increased apoptosis susceptibility of FIV-infected cells and might be involved in FIV-specific T cell depletion {Mizuno, et al. 2001}. Whether TNF-α production by FIV-specific T cells is beneficial or detrimental for FIV-infected cats still needs to be determined. IL-1 and IL-6 are common cytokines generally produced in the course of infectious processes. In addition to proinflammatory effects, the elevated production of IL-6 may contribute to polyclonal B-cell activation and gammopathies {Flynn, et al. 1994}.

Peak production of IL-1, IL-6 and TNF coincided with periods of depressed immune responses in vivo and the presence of clinical signs. Additionally, high expression of these proinflammotory cytokines coincide with depressed responsivness of PBMC to mitogenic stimulation in vitro {Lawrence, et al. 1995}.

Dysregulation of cytokine responses in FIV infected versus FIV-naïve cats induces inability to effectively fight against secondary pathogens. Observed cytokine profile during secondary opportunistic infections with Toxoplasma gondii {Levy, et al. 1998} or Listeria monocytogenes {Dean, et al. 1998} indicate a loss of the important Th-1 cellular immune response during FIV infection. Although controversed for a long time, it is now overall accepted that the cytokine patterns seen in the course of FIV infection implicate a

dominating Th-2 type immune response, and that attempts to elaborate effective vaccines should concentrate on supporting and enhancing Th-1 orientated T helper response.

2.2.3.5. Antibody Responses:

FIV infected cats develop a strong immune response against the viral *gag* and *env* proteins. Moreover, parts of the surface glycoprotein, the transmembrane protein and *gag* proteins are considered major B-cell epitopes. Cats infected experimentally can seroconvert already 2-3 weeks post-challenge, and antibodies against envelope proteins seem to arise first, followed by a response against *gag* elements {Egberink, et al. 1992}. Antigen stimulation of infected B-cells is increased compared to non-infected cells, and a polyclonal gammopathy directed against non-viral proteins may be seen in FIV infected cats as early as 6 weeks post infection {Flynn, et al. 1994}.

The development of virus neutralizing (VN) antibody is one of the most effective host defense mechanisms, and persistence of FIV infection in vivo in spite of high VN titers has been thoroughly studied. The V3-V5 areas of the env gene are known to code for neutralizing epitopes of FIV. It has been shown that single amino acid substitution mutations in the envelope glycoprotein, within or outside the VN epitopes, may confer resistance to virus-neutralizing (VN) monoclonal or polyclonal antibody {Siebelink, et al. 1995}. In vitro sensitive FIV strains have shown reversion to neutralization resistance due to mutations in V4 and V5 regions of the env gene, after passage in vivo for 4-15 months. This mutated phenotype appears to be essential for survival and persistence of virus in vivo {Bendinelli, et al. 2001}.

VN antibody titers increase progressively during the first 30 weeks post inoculation and remain at high titers thereafter for 7 years of observation {Inoshima, et al. 1996}. Cross-clade neutralization is common {Inoshima, et al. 1998}, indicating that important neutralization-inducing epitopes are universally shared in spite of high antigenic diversity in FIV strains prevailing in the field.

2.3. Epidemiology:

2.3.1. Viral Clades:

FIV is endemic in cats throughout the world. Five distinct clades of FIV (A-E) have been identified based on greater than 15-30 % variability in envelope amino acid sequence. The majority of viruses identified to date belong to either clade A or clade B. The prototype virus, FIV-Petaluma, discovered in 1986 by Pedersen and colleagues, is attributed to clade A, a group of strains significantly less diverse than clade B, and containing fewer genomic mutations, suggesting more recent, less host-adapted strains {Bachmann, et al. 1997}. Clade A viruses are present worldwide with a predominance in the western United States, northern Japan, Germany, South Africa. Also distributed worldwide, clade B viruses have

been more consistently identified in eastern Japan, Italy, Portugal, and the central and eastern United States. With the exception of northern Taiwan, detection of clade C FIV remains uncommon, and has often only been attributed to single animals or small groups of cats from Vancouver, Munich and Japan {Sodora, et al. 1994}. Clade D includes several viruses characterized mainly in western Japan {Kakinuma, et al. 1995}. Finally, two Argentine strains comprise clade E {Pecoraro, et al. 1996}.

Similarly to other lentiviruses, FIV is very susceptible to evolutionary genetic alterations and variability in phenotype as a consequence. Genetic diversity both due to evolution of the virus within an infected individual and by recombination following dual infection have been described for FIV. Indeed, intra-individual *env* variation is known to be the major determinant of viral diversity in virus isolates {Kyaw-Tanner and Robinson et al. 1996}. Recent studies have however demonstrated stable maintenance of the V3-V5 env gene encoding neutralizing epitopes for 1-2 years after infection {Motokawa, et al. 2005}. Co-infection with two strains, although uncommon, has been successfully established under experimental conditions {Okada, et al. 1994} and its occurrence confirmed in naturally infected animals {Bachmann, et al. 1997}. It has also been demonstrated that biological behaviour and pathogenicity differ from one isolate to another {de Monte, et al. 2002}. Thus, properties identified for a specific strain cannot be generalized, and regular phylogenetic studies allowing evaluation of evolutionary viral adaptation to the host are a prerequisite when developing new prophylactic measures or treatments.

2.3.2. Host Range:

Domestic cats (felis catus) are the natural hosts for FIV. However, cross-reactive antibodies have been detected in over half of the species of the family Felidae {Olmsted, et al. 1992}. Transmission of FIV from domestic to non-domestic cats has been reported {Nishimura, et al. 1999}, but the majority of FIV-reactive antibodies in non-domestic cats are induced by other FIV-related species-specific lentiviruses, as described for the puma, *Puma concolor* {Biek, et al. 2003}, the lion, *Panthera leo* {Brown, et al. 1994}, and the Pallas' cat, *Otocolobus manul* {Barr, et al. 1995}. These viruses are known to be older and more stable than FIV, and recent studies surprisingly indicate that they diverge greatly in amino acid sequences, host cell susceptibility, receptor affinity, neutralizing antibody binding sites, and pathogenicity, when compared to FIV {Smirnova, et al. 2005}.

Interestingely, for many years it was believed that infection by the equivalent of FIV (FIVple, standing for Panthera Leo) of free ranging lions would not have any adverse effect on their health. Now, a recent publication has demonstrated that in some individuals

infection with FIV ple can lead to clinical, immunological, and pathological deterioriation compared with non infected peers {Roekle, et al. 2009}.
Non-domestic cat lentiviruses can also establish persistent infection in domestic cats; however clinical disease is frequently absent {VandeWoude, et al. 1997}. Interestingly, infection of domestic cats with a non-domestic lentivirus generates humoral and cell-mediated immune responses and thus provides partial protection from disease induced by superinfection with FIV {VandeWoude, et al. 2003}.
Infections of human cell lines with FIV result in proviral integration {Ikeda, et al. 1996} and at least two FIV strains have been able to infect human PBMC and macrophages in vitro {Johnston, et al. 1999}. Moreover, experimental FIV infection of cynomolgus macaques resulted in loss of CD4+ cells and weight loss {Johnston, et al. 2001}. Cross packaging of HIV-1, SIV and FIV RNA have been demonstrated, suggesting that co-infection could result in stable recombinant viruses {Browning, et al. 2001} However, these findings must be considered with precaution , and should not be extrapolated to suggest that human infection can occur. Replication of FIV has to date not been observed in human cells, and lack of human seroreactivity despite significant exposure to infected cats indicate very low potential for zoonosis among healthy adult humans {Butera, et al. 2000}.

2.3.3. FIV prevalence:

Several seroepidemiological surveys have revealed an overall seroprevalence worldwide of 11.04 % among both healthy and sick cats screened in North America, Asia, Europe and Oceania {Kanzaki, et al. 2004}. It is important to keep in mind that prevalence rates vary greatly by region, lifestyle, health status and gender of the cats tested. Furthermore, these studies are likely to underestimate reality, since they often don’t take into consideration that some of FIV infected cats are seronegative (see table 1).

2.4. Clinical Aspects:

Similarly to HIV infection in humans, FIV causes progressive immune deterioration in domestic cats. Since individuals become persistently infected, complete recovery is not possible, and infected cats eventually die of the disease. Although all categories of domestic cats may become infected, susceptibility to FIV infection and disease progression are inversely related to age {Pedersen, et al. 2001}.
During the acute phase of infection, the cat usually shows only little or no symptoms at all. Typical are low-grade fever, generalized lymphadenopathy eventually accompanied by slight lethargy {Pedersen, et al. 1989}. Unfortunately, owners often fail to notice these rather mild and unspecific signs, and clinical suspicion of disease is only assessed after greater progression of the infection. Evaluation of lymphadenopathy in FIV infected cats

has revealed histopathological alterations in the lymph nodes of the hindlimb, forelimb and head, in decreasing order of severity, with little evidence of involvement of alimentary tract associated lymph nodes {del Fierro, et al. 1995}. The popliteal lymph node is thus considered a good indicator for assessment of lymph node status in FIV infection.

The clinical signs of the ARC and AIDS stages are diverse in nature because symptoms due to primary viral infection are frequently overlapped by those of an array of secondary infections {Lappin, et al. 1995}. Slow but progressive weight loss is common, with severe wasting occurring late in the disease process. Fever of 40˚C or greater is often present. Typical are also a loss of appetite or evidence of pain while eating, caused by gingivitis and stomatitis. Chronic, non-responsive, or recurrent infections of the skin, alimentary tract upper respiratory tract and eyes are often observed {Yamamoto, et al. 1989}. Infection may lead to abortion of kittens or other reproductive failures in pregnant queens {Weaver, et al. 2005}. Encephalopathy –linked neurological abnormalities, although rather rare are occasionally observed. Dementia, twitching movements of the face and tongue, loss of bladder and rectal control, cognitive and behavioral alterations and compulsive roaming have all been recognized in FIV-infected cats. Seizures, ataxia and intentions tremor has also been described {Podell, et al. 1993}. Renal involvement in FIV disease has been described as a frequent occurrence and direct consequence of viral infection. Moreover, presence of immuncomplexes in renal tissue suggests that immunological processes in the course of FIV infection play a role in the pathogenesis of renal damage {Poli, et al. 1995}. Interestingly, no link has been established between FIV infection and lower urinary tract disease, a relatively common disorder in adult cats {Barsanti, et al. 1996}.

The role of FIV in tumorigenesis is still quite controversial. As reported for SIV and HIV, it has been postulated that occurrence of lymphoma could be related to infection in FIV seropositive cats {Poli, et al. 1994}. The majority of presumed FIV associated lymphomas are of B-cell blastic phenotype and usually occur at a single extranodal site {Callanan, et al. 1996}. Examination for FIV provirus in tumorous cells has however been inconsistent in both experimentally and naturally infected cats, and the amount of cases remains small. Lymphoid malignancies, myeloproliferative diseases, and several carcinomas and sarcomas have also been more frequently detected in FIV infected, FeLV-naïve cats, suggesting a potential association between FIV and malignancy.

Secondary infection during ARC and AIDS stages of disease can be due to various microorganisms which take advantage of the weakened immune system, including other viruses, bacteria, parasites and fungi. Among predisposition to other parasitical infections related to FIV disease, toxoplasmosis, as an opportunistic infection in HIV-infected humans, is of high interest. Primary infection of cats with FIV markedly enhances their

susceptibility to a secondary *Toxoplasma gondii* infection {Davidson, et al. 1993}. Furthermore, FIV infected cats develop higher *T.gondii* IgM antibody levels and increased replication rate of *T.gondii* {Lappin, et al. 1993}.
Another frequently studied association is that of possible relationship between FIV and FeLV infections. Seroepidemiologic surveys have indicated that dual infection is not uncommon, and that cats infected with both viruses tend to have more severe disease course and die sooner than monoinfected animals {Cohen, et al. 1990}. Moreover, FeLV infection has proven to enhance FIV infection in vitro and in vivo by facilitating expression and spread of FIV in the body, leading to faster decline of the immune system and earlier signs of infection ({Pedersen, et al. 1989}.
Certain changes in blood and bone marrow parameters are consistently observed in FIV infected animals. In the primary stage, a leucopenia mainly due to an absolute neutropenia is frequently observed {Yamamoto, et al. 1988}. Towards the end of the disease, in ARC and AIDS stages, common findings include anemia, lymphopenia, thrombocytopenia, neutropenia in blood, as well as hyperplasia of individual cell lineages and dismorphic alterations in bone marrow {Shelton, et al. 1990}. In addition, several significant alterations in clinical chemistry have been noted as of the ninth month post infection: deviations from the reference ranges were observed for glucose, serum protein, gamma globulins, sodium, urea, phosphorus, lipase, cholesterol, and triglycerides {Hofmann-Lehmann, et al. 1997}. However, these abnormalities are not pathognomonic for FIV infection and they are therefore not reliable in characterization of disease progression. Clinical markers representing more effectively the impairement of the immune system and allowing prediction of clinical outcome are CD4+:CD8+ lymphocyte ratio and plasma viral load {Goto, et al. 2002}.

2.5. Diagnostics:

In addition to multiple haematological, serum biological and cytological bone marrow abnormalities which may lead to suspicion of an FIV infection, several elaborated laboratory tests allow the diagnosis.
ELISA and immunochromatography tests are available in kit form for use in private veterinary clinics, and are usually used as screening tests. Several variants are commercially available and detect antibodies against the FIV transmembrane protein or the transmembrane protein combined with gag proteins. As cats do not recover from FIV, a direct correlation exists between the presence of antibodies and virus infection. Antibodies to FIV are usually detected for the first time between 10 and 60 days after exposure, and during the rest of the infected animal's life. Clinical signs can however

appear before seroconversion and some cats do not exhibit detectable levels of antibodies in their blood for weeks and months of infection {Dandekar, et al. 1992}. Moreover, antibody levels can fall below detection level in the final stage of disease {Pedersen, et al. 1989}. These facts lead to occurrence of false-negative reactions. In addition, the specificity of ELISA tests is unfortunately not optimal. Positive results should always be considered in relation to prevalence, and confirmed by a second test in healthy or low-risk cats {Hartmann, et al. 1994}. Confirmatory tests, performed commonly at diagnostic laboratories, include Western Blot or Indirect fluorescent antibody immunoassays (IFA). The major advantage of Western Blot immunoassays is the detection of multiple antibody specificities in one reaction against various viral proteins. Methods were described rapidly after discovery of FIV {Lutz, et al. 1988a, Lutz, et al. 1988b, Gruffyd-jones, et al. 2001, Bennett, et al. 989}. A densitometric analysis of Western Blots enabling to quantify the antibodies against FIV proteins have also been described {Calandrella, et al. 2001}. Detection of antibodies with IFA is based on the binding of specific antibodies in diluted serum samples to antigen expressed by FIV infected T-lymphocyte-enriched peripheral blood mononuclear of CRFK cells as substrate. In a second step, bound antibodies are stained with fluorescein-labelled anti-cat antibodies and made visible with the fluorescence microscope {Reid, et al. 1991}.

It is worthwhile mentioning here that kittens can have detectable colostrum-derived antibodies for several months. Therefore, only kittens older than 6 months with positive results in both ELISA and second confirmatory tests can be considered infected.

ELISA tests detecting antibodies of various specificities have been elaborated over the years. It has been demonstrated that over 90 percent of FIV infected cats establish an antibody response to the reverse transcriptase enzyme, which displays increasing in vitro inhibitory effects over time post-infection. Unfortunately, significant concentrations of reverse transcriptase inhibiting antibodies are detected only 1 to 2 years after infection, rendering use of this test rather irrelevant in routine diagnostics {Fevereiro, et al. 1991}. In 1992, Furuya developed an ELISA system for detection of antibodies to FIV *gag* protein in cat sera {Furuya, et al. 1992}. They thereby observed increases of the antibody titers to FIV *gag* protein in all studied cases, already at an early stage of infection. However, the establishment of ELISA systems using recombinant surface (SU), transmembrane (TM) and capsid (CA) antigens of feline immunodeficiency virus (FIV) and comparison of their individual efficiency showed highest diagnostic sensitivity (98%) and specificity of 97 % for detection of TM antibodies. Furthermore, observation of an early and marked increase of TM antibodies indicates importance of TM in ELISA testing for FIV {Calzolari, et al. 1995}.

Detection of a FIV antigen in infected cats is of low interest in routine diagnostics due to the often insignificant levels of virus in plasma during the asymptomatic phase of disease. For research purposes, the establishment of a p24 antigen capture ELISA system has shown however convincing potential for monitoring FIV replication in vitro {Kashiwase, et al. 1997}.

Various cell culture techniques have also been established to identify cellular or plasma-related viremia. Thus, isolation and in vitro stimulation of peripheral blood mononuclear cells (PBMC) with subsequent detection of produced viral antigen allows the assessment of cellular infection. With the intention to simplify such experiments, {Guiot, et al. 1995} developed an assay allowing direct culture of small amounts of whole blood (100 µl), followed by detection of FIV core gag antigen released in culture supernatants. Avoiding the hassle of leukocyte separation and lymphocyte purification procedures, this technique offers many advantages and shows convincing reproducibility. Moreover, several FIV-sensitive cell lines allow assessment of infectious virus in plasma of infected cats (see 2.1.2.2.). A T-lymphoblastoid cell line obtained from the PBMC of a specific pathogen free cat and designated MBM was even reported to exhibit a lytic cytopathic effect in vitro upon FIV infection {Matteucci, et al. 1995}.

The development of polymerase chain reaction (PCR) assays represents a significant step for the identification of the proviral and viral forms of FIV in routine diagnostics, as a complement to common methods, as well as in research areas. Detection of provirus by a double PCR method using regions of the *gag* gene as target sequences was described as early as 1992 {Hohdatsu, et al. 1992 ; Rimstad, et al. 1992}. Soon after, Matteucci and colleagues established an assay allowing assessment of FIV RNA {Matteucci, et al. 1993}. Although sophisticated and complex, these early PCR methods often failed to harbour satisfying sensitivity levels. The need in further understanding of the pathogenesis and pressure in the development of new markers of disease progression induced establishment of quantitative PCR methods. In this way, two groups described an assay for the measurements of proviral DNA copies of FIV {Inoshima, et al. 1995; Allespach, et al. 1996}. Furthermore, using in vitro synthesized RNA derived from the *gag* region of the FIV genome as competitive control, {Vahlenkamp, et al. 1995} succeeded in quantifying FIV RNA from plasma of infected cats in a conventional PCR assay.

The latest evolution in quantification of FIV DNA and RNA is the TaqMan fluorogenic real-time detection system {Leutenegger, et al. 1999}. This improved PCR method is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of a fluorescent dye-labelled probe during the amplification cycles. The intensity of

fluorescence is then measured in a dedicated instrument. A more detailed characterization of the TaqMan system is described under chapter III.
Further characterization of FIV pathogenesis in vivo relies on the localization of virus to specific cells in tissues, enabling detection of host-virus interactions at different time points in disease. FIV was most often revealed in tissue sections by *in situ* RNA hybridization. Unfortunately, tissue digestion steps required for *in situ* RNA hybridization often destroy protease-sensitive cell-specific antigens, limiting the number of markers available to identify the cells infected. Identification of FIV-specific proteins by immunohistochemistry circumvents the need for protease digestion steps and RNase-free protocols. However, both conventionally used mouse monoclonal and rabbit polyclonal antibodies lack specificity and sensitivity in binding FIV in tissue sections. In 2002, Rogers and colleagues established an immunohistochemical protocol using high-antibody-titer serum from cats chronically infected with FIV-PET. This method includes labeling of the anti FIV antibodies with protein A followed by avidin conjugated peroxidase. In complement to other *in situ* methodologies, this assay allows detection and quantitation of virus in tissues from cats infected with FIV clade B or clade C. Such steps in diagnostic research contribute to the further understanding of important processes concerning tropism and replication kinetics of FIV infection in vivo {Rogers, et al. 2002}.

2.6. Treatment:

There is unfortunately up to date no treatment allowing effective elimination of FIV in an infected cat. As a consequence, therapy of FIV-associated disease is mostly supportive, with the objective of increasing the patient's life quality and length. In addition to cause-related treatments such as antiviral and immune stimulation drugs, immunodeficient cats in the late stages of disease may require appropriate long-term antimicrobial therapy or multiple treatment periods to fight secondary opportunistic infections.
Various approaches in treatment against FIV have been experimented. Over the years, researchers have been greatly inspired by the progress in treatment of HIV. Indeed, the most promising results to date include drugs interfering directly with retroviral replication, derived from HIV research.
Four classes of antiretroviral agents are currently available for the treatment of HIV infection: nucleoside and nucleotide analogs inhibit RT, (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs) and fusion inhibitors. The highly active antiretroviral therapy (HAART) commonly administered to HIV infected patients, consists of a combination of three drugs from one or more of the above mentioned groups. Typically, the therapy includes one nucleoside analog, one protease inhibitor and either a

second nucleoside analog or a NNRTI. The major problem encountered during antiretroviral therapy is the appearance of resistance to treatment due to mutative adaptation of the virus. Chronically HIV infected patients having received various combinations of HAART over several years may develop multi-resistant strains of the virus, rendering treatment ineffective. As a consequence, although a wide range of drugs exist, there is a constant need for new therapeutic strategies.

Treatment effectiveness of all four classes of antiretroviral agents and in various combinations has been experimentally evaluated for FIV.

Nucleoside analogs are also referred to as RT inhibitors (NRTIs). Their target is the retroviral enzyme reverse transcriptase. Acting as alternative substrates, they compete with physiological nucleosides, differing from them only by a minor modification in their ribose molecule. The incorporation of nucleoside analogs aborts DNA synthesis by the reverse transcriptase, as phosphodiester bridges can no longer be built to stabilize the double strand. NRTIs are not HIV-specific inhibitors as they are broadly effective to other lentiviruses as well, including FIV {North, et al. 1990}. Azidothymidine (AZT), a thymidine analog also designated zidovudine, was the first antiretroviral agent to be put on the market for HIV treatment. Early studies still tested AZT as monotherapy. In 1993, Meers and colleagues described a significant reduction of plasma virus titer by zidovudine treatments in asymptomatic cats, begun 24 h post infection and continued for 4 weeks {Meers, et al. 1993}. However, the virus titers in PBMC were high for the whole duration of the experiment. Furthermore, in a study evaluating long-term effectiveness of AZT compared with conventional symptomatical therapy in FIV-seropositive diseased cats, AZT led to total recovery from clinical symptoms in six of nine FIV-seropositive cats 4-6 weeks after the onset of therapy. Although all the FIV-seropositive cats treated symptomatically in this study responded well to antibiotics and immunomodulators within as little as 10-14 days, recurrence of severe clinical symptoms was noticed in most cases {Hart, et al. 1995}. In the interest of diminishing undesired adverse effects of AZT monotherapy, such as anemia and hypoproteinemia, researchers have more recently tested the effect of NRTI combinations. Lamivudine (3TC) is a well tolerated cytosine analog in clinical use against HIV. In vitro studies utilizing AZT alone, lamivudine (3TC) alone or AZT/3TC combination indicated that simultaneous treatments of AZT and 3TC generated additive to synergistic effects in primary PBMC, but not in chronically infected cell lines. Similarly, AZT/3TC combination induced a significant delay in infection and seroconversion in unprotected cats, but had no anti-FIV activity in chronically infected cats. AZT/3TC treatment seems therefore effective for prophylaxis but not for therapeutic use in chronically FIV-infected cats {Arai, et al. 2002}. Stampidine is the latest nucleoside analog prepared for treatment

of HIV. It is said to be much more potent than other anti-HIV NRTI agents and seems active against phenotypically and/or genotypically NRTI-resistant HIV strains. Antiretroviral activity of stampidine in cats chronically infected with FIV has also been studied. Notably, a single oral bolus dose of stampidine resulted in a significant decrease in the FIV load of circulating PBMC in five of six FIV-infected cats {Uckun, et al. 2003}. Its antiretroviral properties in NRTI resistant HIV strains, together with its favorable animal toxicity profile, pharmacokinetics, and in vivo antiretroviral activity in FIV-infected cats, renders stampidine a promising new NRTI compound.

Similarly to the nucleoside analogs, the target enzyme of non-nucleoside reverse transcriptase inhibitors (NNRTIs) is the reverse transcriptase (RT). In contrast to the NRTIs, they are not "false" building blocks, but rather attach directly and non-competitively to the enzyme, at a position in close proximity to the substrate binding site for nucleosides. The resulting complex can thus bind fewer nucleosides, slowing down significantly the DNA synthesis rate. In contrast to the nucleoside class of inhibitors, NNRTIs are highly specific for HIV-1, and they are not active against HIV-2 or any other retrovirus, including SIV and FIV. Indeed, despite the high similarity of the NNRTI target sequence between HIV-1 RT and FIV RT, no inhibitory effect of NNRTIs against FIV RT has ever been reported, even at drug concentrations that are significantly higher than those required to fully suppress HIV-1 RT activity {Auwerx, et al. 2002}. With the objective to map the determinants of the lack of susceptibility of FIV-RT to anti HIV-1 NNRTIs, {Auwerx, et al. 2004} and colleagues (2004) then constructed a variety of chimeric FIV-RTs, equipping this enzyme with the amino acids that have been proven to engender susceptibility of HIV-1 RT toward NNRTIs. This study revealed that FIV-RT must be profoundly different from HIV-1 RT in terms of structure and/or flexibility, so as to prevent its interaction with NNRTIs. Thus, to date, researchers have not yet succeeded in either transforming FIV-RT to acquire susceptibility to the inhibitory effects of the NNRTIs, or elaborating a NNRTI which efficiently binds and inhibits FIV-RT.

The retroviral protease (PR) cuts the viral gag-pol polyprotein into its functional subunits. Thus, inhibition of the protease, preventing appropriate splicing and maturation of essential structural proteins, leads to the release of virus particles that are unable to infect new cells. With knowledge of the molecular structure of the protease encoded by the human immunodeficiency virus, the first protease inhibitors were designed in the early nineties. However, the emergence of resistant strains, in which the sequence of the viral PR is altered in a way that leads to the impairment of inhibition, has grown rapidly problematic. Again, despite high structural homology of both proteases, FIV PR demonstrates poor binding of inhibitors in clinical use against HIV-1 PR. Several studies have shown that FIV

PR contains, at equivalent positions, amino acid residues that are identical to those found in drug-resistant forms of HIV-1 PR. The protease structure of FIV was then used as a model for the development of a series of protease inhibitors with broad efficacy. The most potent FIV PR inhibitor, designated TL-3, was able to block nearly 100% of virus production in an acute infection against HIV, FIV, and SIV ex vivo. Furthermore, it was not toxic to cells, and there was no sign of resistance development by the different viruses even after 2 months of culture {Lee, et al. 1998}. In vivo, TL-3 treatment engendered modest lowering of viral loads and greater survival rates in treated symptomatic animals at eight weeks post infection with a highly pathogenic FIV-C isolate {de Rozieres, et al. 2004}. Moreover, early TL-3 treatment was shown to effectively counteract FIV effects on the central nervous system (CNS) of infected cats as well as eliminate FIV-induced changes in the CNS {Huitron-Resendiz, et al. 2004}. Although continued treatment is required to lower average viral loads and to maintain unimpaired CNS function, TL-3 seems to have a considerable therapeutic effect against FIV infection.

Inhibition of FIV entry into host cells provides an exciting new and emerging approach for the development of anti-viral therapies. The identification of the peptide sequences responsible for gp120/receptor binding allows the creation of a class of pharmaceuticals called fusion inhibitors, whose mechanism of action is to competitively antagonize virus binding. In 1996, Lombardi and colleagues, screened 20- to 23-mer peptides covering the entire *env* gene of FIV and found especially potent in vitro antiviral activity associated with a peptide referred to as peptide 59, derived from a region located proximally in the ectodomain of the transmembrane glycoprotein (TM gp). Unfortunately, peptide 59 did not appear promising as a therapeutic strategy for naturally occurring infections since it could only inhibit tissue culture-adapted FIV in fibroblastoid cells (CrFK), but not a primary isolate in feline lymphoid cells. Because the activity of peptide 59 mapped to a short sequence containing three conserved Tryptophan (Trp) residues, further analyses were carried out with a peptide of eight amino acids, designated C8, comprising such a Trp motif {Lombardi, et al. 1996}. Peptide C8, although rather unstable due to its reduced size, exerted a powerful antiviral effect in vitro on all the FIV isolates tested, and this activity was dependent on an intact Trp motif {Giannecchini, et al. 2003}. Further studies have demonstrated that, the C8 retroinverso analogue (riC8) possesses similar inhibitory potential and a much increased stability, maintaining its concentration unchanged for at least 24 h in cat serum in vitro. Most exciting are the results of a short-term monotherapy experiment in chronically FIV-infected cats showing that riC8 is well tolerated and also has substantial antiviral activity in vivo {Giannecchini, et al. 2005}.

Relatively new treatment perspectives focus on modulation of the host immune system, exploring the potential of various immunostimulatory cytokines and antioxidants. Immunostimulatory agents are probably the most widely used medications in FIV-infected cats.

In addition to their immunomodulatory effect, both type I and type II interferons also exhibit a direct antiviral effect by inducing a general antiviral state in cells, that protects them against virus replication. Strong inhibitory activity of human as well as feline type I IFN against FIV replication has been reported with *in vitro* treatment of a feline T cell line and feline PBMC with recombinant human interferon alpha (rHuIFN-*α*) or its corresponding feline interferon, the recomnbinant feline interferon Ω (rFeIFN Ω) {Tanabe, et al. 2001}. As interferons are species-specific, feline and human interferons clearly differ in both antigenicity and antiviral efficacy in feline cells. Thus, rHuINF-α showed insignificant effect on peripheral blood lymphocyte subsets in FIV infected cats, and was clearly ineffective in immunodeficient animals, who exhibited severe lymphocyte depletion. Although monotherapy was thus excluded, rHuINF-α seemed however to demonstrate some clinical benefits with no side-effects when used in complement to antibiotic and symptomatic therapy {Riondato, et al. 2003}. In contrast, rFeIFN Ω demonstrated significant therapeutic effects and increase in survival rate of cats with clinical signs associated with FeLV infection and FeLV/FIV coinfection {de Mari, et al. 2004}.

The results of several studies concerning effect of human type II interferon (HuIFN-γ) on HIV replication remain to date controversial, however FIV replication seems not to be inhibited by recombinant feline interferon gamma (rFeIFN- γ) in feline PBMC, and rather enhanced in the FetJ feline T-cell line {Tanabe, et al. 2001}. This finding may be explained by the mostly indirect effects of IFN- γ in vivo, modulated by cytotoxic T cell and NK cell responses to virus-infected cells.

An additional cytokine, the recombinant human granulocyte colony stimulating factor (rHuG-CSF), was recently evaluated for its antiviral effects in the course of FIV disease. Similar to its use in humans, rHuG-CSF can be used to increase the production and functional activity of neutrophils in animals that have infectious disease. However, these drugs are not veterinary-labeled products and the potential risks must be considered in relation to the therapeutic benefits for veterinary use. Overall, rHuG-CSF treatment increased neutrophil counts in FIV-infected cats without affecting the infection status. Furthermore, due to production of neutralizing antibodies to rHuG-CSF, and the possible development of cross-neutralizing antibodies to recombinant feline G-CSF, long term treatment with rHuG-CSF may induce severe adverse reactions and is not recommended in cats {Phillips, et al. 2005}.

Another explored route was the therapeutic potential of several immunosuppressive drugs on FIV. Indeed, as immune activation may lead to increased FIV replication, an attractive treatment hypothesis has been to suppress the immune system in an attempt to interfere with viral replication and apoptosis of host cells. Although glucocorticoids were shown to enhance the course of infection {Barr, et al. 2000}, two potent immunosuppressive drugs, Cylosporine A and Tacrolimus, commonly utilized in preventing graft rejection following organ transplantation, have proved to protect cells against apoptosis and to decrease virus production in both acutely and chronically infected cats {Mortola, et al. 1998}. Use of both immunosuppressants and immunostimulants in the therapeutic setting shows the clear discrepancy between scientific knowledge and hope, and highlights the current difficulties encountered in further development of drugs suitable for treatment of FIV.
Despite numerous studies, clinical use of antiviral drugs is still not very common in veterinary medicine. Many antiviral drugs that have been experimentally tested never appear on the market due to high toxicity. With the exception of the new feline interferon-ω that is now on the market in some European countries and Japan, no antiviral drugs are licensed for veterinary medicine. Therefore, human drugs such as AZT have to be used in animals.

2.7. Prevention:

2.7.1. General:

Although ethically questioned in areas of low FIV prevalence, keeping cats indoors to avoid contact with homeless, feral, abandoned or stray cats is the most effective mode of prevention against infection. Outdoor cats should ideally be neutered, in order to diminish territorial behaviour and risk of transmission during mating. Additionally, testing of new cats before introduction in a FIV sero-negative household is crucial. Infected cats should be housed indoors at all times to avoid contamination of FIV-naive cats and to lessen their own risks to acquire opportunistic infections. Finally, kittens of FIV-infected queens should not be allowed to nurse in order to avoid transmission by ingestion of milk.

2.7.2. Vaccination:

Vaccine development is essential to protect the pet cat population from FIV infection and to control the worldwide prevalence within reservoir stray cat populations. Furthermore, promising FIV vaccine designs may serve as models for the further deveplopment of effective HIV vaccines. The development of lentiviral vaccines remains however a real challenge, due to the viral affection of important host immune components. A healthy immune system is indeed crucial for efficient vaccine immunity. Cellular tropism of FIV for

T and B cell populations as well as for the monocyte /macrophage system renders the development of an efficacious product extremely difficult.

In addition to their complex pathogenesis, lentiviruses are known for their potential to mutate and their diversity in the field. The various FIV subtypes differ in antigenic properties recognized by the immune system, which are, as a consequence, also targeted by vaccine studies. The development of a vaccine which harbours potency against several, or ideally all subtypes of FIV, is crucial to clinical utility in the field, and remains an ongoing challenge.

Efficacious lentiviral vaccine development has indeed revealed itself particularly tricky. Many factors influence greatly the outcome of individual studies, and it is crucial to take them into consideration when criticizing the different attempts elaborated over time. Overall, technical differences in vaccine inactivation procedure and composition, such as cell types used for growing vaccine virus, vaccination schedule, adjuvant used, vaccine doses, and variable virulence between FIV strains have produced conflicting results over time. Analysis of some important influencing factors in vaccine studies is detailed below.

2.7.2.1. Choice of Adjuvant:

An important facet of vaccine production is the selection of an appropriate adjuvant. Although these substances have no specific antigenic effect, their presence in the composition of a vaccine will help to induce a Th1 response, in addition to stimulate antibody production.

In the search of vaccine for diseases with complexe pathogenesis such as lentiviral infections, the conjunction of an adjuvant may obviously play a determinig role in the success of the immunization trial. Different adjuvants are chemically highly heterogenous and affect the immune system in various ways. Their mode of action generally consists in the formation of a gradually-released depot of antigen at the site of inoculation, the presentation of antigen and stimulation of immunocompetent cells, as well as the production of various cytokines and immunoglobulins. Due to the resultant hyperactivation of the host's immune system, they may however induce variably serious side effects. In cats, the development of vaccine related fibrosarcoma, an especially malignant cancer originating from fibrous connective tissue at the site of vaccine injection, has grown to a serious concern in the past years. There is evidence that adjuvants play an inciting role in the formation of these deadly tumours {Rolline, et al. 1999}. The choice of an adjuvant thus reflects a compromise between requirement for adjuvanticity and an acceptable level of adverse reactions.

Freund adjvant (CFA) was the first substance used successfully as adjuvant. CFA is mineral oil, composed of inactivated and dried mycobacteria, usually *Mycobacterium*

tuberculosis, which -after being emulsified with antigen in aqueous phase- stimulates cell-mediated immunity leading to the potentiated production of certain immunoglobins. Due to its toxicity, its use in humans is forbidden, and these are currently a number of guidelines associated with its use in animal research. While usually effective, it may indeed induce undesirable side effects such as inflammation, induration, pain and necrosis at the injection site in mammals such as goats these facts have led to refinement of its use and development of alternatives.

In FIV vaccine trials, a variety of alternatives to Freund's adjuvant have been tested. In this way, incomplete Freund's adjuvant (IFA), lacking killed mycobacteria which represent the most inflammatory component of CFA, was initially used for boosting immunizations subsequent to CFA. It may also serve for initial immunization, particularly in combination with a strong antigen. Similarly, the main chemical structure of the mycobacterium in CFA, peptidoglycan muramyl dipeptide (MDP), provides the desired adjuvant activity and its use decreases significantly the disadvantages encountered with CFA. When MDP was incorporated into incomplete Freund's adjuvant it was found to replace completely the adjuvant activity of the mycobacterium. Moreover, MDP was also found to have adjuvant activity when used alone. Although its pyrogenic potential remains a limiting factor in its use as adjuvant, MDP has been used in various forms and combinations in FIV vaccine studies. Several oil-in-water emulsions represent another class of widely used adjuvants. The Ribi adjuvant contains detoxified endotoxin with mycobacterial cell wall components. It presents very low toxicity and is as convenient to use as Freund's complete adjuvant, due to its low viscosity. Similarly, Syntex Adjuvant Formulation (SAF) is a stabilized oil-in-water emulsion known to activate complement by the alternate pathway. SAF was used quite successfully in early vaccine studies combined to various forms of MDP {Tellier et al. 1998}.

Mineral compounds are also of common use for supplementation of vaccine antigens. Aluminium hydroxide, for example, allows particularly slow release of the antigen at the injection site, prolonging the time for interaction between antigen and antigen-presenting cells and lymphocytes. Its use in cats however has been controversial, as this agent has been speculated to play a role in the induction of lethal tumours at the injection site {Usinger et al. 1997}.

Plant derived chemicals have also been tested for their adjuvant effect for FIV vaccines. Quil A, a highly refined form of saponin, was also shown to stimulate both cell-mediated and humoral immune responses. Additionally, it serves as the main component of immune stimulating complexes called ISCOMs, relatively stable but non-covalently-bound complexes of Quil-A, cholesterol and amphipathic antigen. The main characteristic of

ISCOM-associated antigen molecules is that they are transported to the draining lymph nodes and thus do not deposit at site of injection. Furthermore, very low quantities of antigen can elicit a significant immune response, thus rendering the use of ISCOMs an interesting approach to stimulation of the host immune response towards amphipathic antigens. ISCOMs have only been used in veterinary vaccines, partly due to their haemolytic activity and some local reactions, all reflecting the detergent activity of the Quil-A molecule.

A new class of adjuvants has arisen with the introduction of DNA vaccination, which enables codelivery of specific antigens and molecular adjuvants, such as cytokines or unmethylated CpG oligonucleotide motifs, as an attempt to favourably alter initial immune events in infection. Indeed, the earliest antiviral defenses are innate, and provide the basis on which the subsequent adaptive immune response is built. Further study of lentiviral pathogenesis has led to characterization of unfavourable alterations or insufficient host response soon after the onset of infection, indicating the importance of influencing the innate immunity in the development of effective lentiviral vaccines. In this way, coexpression of vaccine antigens with cytokines abnormally produced in the early events of infection, such as IL-12 and IL-16, has indicated promising results. Similarly, CpG motifs, comprised of unmethylated dinucleotides flanked by two 5' purines and two 3' pyrimidines, directly activate monocytes, macrophages, and dentritic cells to secrete IL-12 and IFN-γ. Their utlity has been demonstrated for a variety of antigens, and they initiated convincing results as adjuvants in DNA vaccines {Leutenegger, et al. 2000}. Further characterization of CpG oligonucleotides as well as their effects on the immune system is thouroughly described in the third chapter of this literature overview.

Unfortunately, no independent experiments comparing a wide variety of adjuvants to each other have been carried out so far. Only individual studies which have used several adjuvant combinations allow limited comparison of different adjuvant types and combinations. Thus, at this current stage of FIV vaccine development, there are no general conclusions as to which adjuvant formulation is more effective.

2.7.2.2. Importance of Challenge Methods:

The evaluation of vaccine efficacy should readily take into consideration the challenge conditions. Indeed, the outcome of the study is greatly influenced by features such as quantity and virulence of the strain used, number of subtypes tested, source of the challenge inoculum and challenge route.

Since no vaccine may be able to provide complete protection against a high-dose challenge or a highly virulent strain, these situations require modifications in the standards of judging vaccine efficacy. Generally, vaccines are designed to provide immunity capable

of destroying all traces of virus in the host. Preclinical studies have however demonstrated that protection against disease is easier to achieve than prevention of infection. Efficacy of a vaccine should be based on observed effects on hallmarks of active infection, such as virus load, CD4+ counts, CD4+/CD8+ ratio, and specific FIV antibody titre. Although the achievements of such studies which failed to demonstrate sterilizing immunity are considered insufficient for clinical use, induction of partial immunity in presence of a severe challenge system surely represents a great step towards the finding of an efficacious vaccine.

Many studies indicate efficient protection against infection with a homologous strain. However, due to the genetic variability of the different FIV subtypes, the demonstration of protection against both homologous and heterologous challenges has become a standard feature in FIV vaccine development. Multi-subtype vaccines not only broaden immunity and protection, but seem to have a synergistic protective potential. In this sense, several dual subtype vaccines have been tested for veterinary use against FIV infection. In 2002, the United States department of Agriculture approved Fel-O-Vax FIV, a dual subtype FIV vaccine, for commercial use. More details concerning the first vaccine against FIV ever released on the market are discussed under 2.7.2.4.2.

Vaccine prophylaxis is also influenced by the source of the challenge inoculum. Studies suggest that homologous FIV vaccine protection using in vitro derived inoculum challenges may not provide protection against challenges using in vivo derived inoculum {Pu, et al. 2001}. In vivo derived inoculum consists of plasma or infected cells derived from cats infected with in vivo passaged laboratory isolates, which contain quasi-species of FIV and more closely simulate natural conditions. This approach, however, encounters multiple limitations in inoculum production, achievement of high titres, and increased costs. Furthermore, biological effects of the plasma or the cellular component of the inoculum are more variable among the recipient animals, in comparison to in vitro derived inoculum.

At last, it is important to take the route of challenge into consideration when judging results of a vaccine study. Conventional methods include subcutaneous (s.c), intramuscular (i.m), intraperitoneal (i.p), intravenous (i.v) and mucosal exposures. It is still controversial which route most closely mimics the natural infection route of biting and fighting. However, it is known that i.v and i.p routes readily infect cats, whereas the s.c and mucosal routes require higher challenge doses. Since the level of the FIV exposure during natural transmission remains unknown, the efficacy evaluation using a contact challenge system represents the ultimate test for any commercial FIV vaccine. According to contact studies and epidemiological surveys, contact transmission of FIV requires long-term exposure (months to years) to naturally infected cats, and disease manifestation is not common

during the early phases of natural infection, supporting the view that natural transmission occurs at low doses {Hartmann, et al. 1998}. Although this method most closely mimics natural conditions, questions arise concerning the dose requirements for natural transmission, the virulence of natural occurring populations, and the consequent maintenance of high viral loads in body fluids of different FIV strains, as opposed to laboratory strains. These obstacles obviously question the feasibility of natural transmission studies for commercial vaccine validation, but such studies will become increasingly important to improve the quality of newly produced promising vaccines.

2.7.2.3. Immune Correlates of Protection and Passive Immunity:

Passive transfer of protection plays a less important role in FIV than in HIV disease, where preventing infection after accidental exposure to the virus or interrupting transmission of virus from mother to child demands efficacious post-exposure prophylaxis possibilities. Although clinical use of passive immunization remains insignificant in the case of FIV, several studies concerning passive immunity have allowed further determination of important mechanisms in FIV vaccine protection.

Hohdatsu, Pu and colleagues {1995} evaluated the role of antiviral antibodies by passive immunization against experimental infection. Cats to whom sera from either FIV-infected or FIV-vaccinated cats had been transmitted, were protected from infection. Furthermore, maternal antiviral antibodies, including VN antibodies, from either infected or vaccinated queens were shown to protect neonatal kittens from FIV inoculation. Thus, maternal antiviral antibodies play a key role in preventing or limiting infection in neonates and vaccinated queens can provide such antiviral immunity {Pu, et al. 1995}.

In another study, cats were either passively immunized with serum antibodies or transfused with peripheral blood cells from FIV-vaccinated cats. In both cases, the immunized animals were protected against an FIV challenge which infected all control cats, suggesting that not only humoral, but also cell-mediated immunity from vaccinated animals can protect naive animals upon experimental FIV challenge {Pu, et al. 1997}. The role of cellular immunity in vaccine protection against FIV infection was further evaluated using adoptive cell transfer studies. It could be demonstrated, that protection mediated by adoptive transfer of immunocytes from vaccinated cats was MHC-restricted, occurred in the absence of antiviral humoral immunity, and correlated with the transfer of cells with FIV-specific CTL and T-helper activities {Pu, et al. 1999}. Thus, both humoral and cellular immunity seem to be able to individually confer protection against homologous challenge and should be, as a consequence, together induced for optimal vaccine protection.

2.7.2.4. FIV Vaccine Trials:

2.7.2.4.1. General information:

Whole virus vaccines, live or killed, constitute the vast majority of vaccines in use at present, in both human and veterinary medicine. Approaches using attenuated live retroviral vaccines are considered to be impractical for clinical trials in humans, due to risk of reversion to virulence. In FIV vaccine trials however, the highest levels of success has been achieved with inactivated whole virus or inactivated infected cell vaccines. Heat, chemicals or irradiation render thereby the virus completely non-infectious. It is important to note that since they are incapable of replicating in the host, inactivated vaccines require larger amounts of antigen.

Recent advances in molecular biology have provided alternative methods for producing vaccines. Technology for growing viruses to high titres in cell cultures has enabled purification of virus and viral antigens. The identification of important peptide encompassing the major antigenic sites of viral antigens allows the production of highly purified subunit vaccines. Virus proteins can be expressed in bacteria, yeast, mammalian cells, and other viruses, and used as recombinant vectored vaccines. An interesting possibility is the production of synthetic peptides closely mimicking specific viral immunogenic sites. The antigens of synthetic peptide vaccines are precisely defined and free from unnecessary components which may be associated with side effects. Overall, the production and quality control of such subunit vaccines is simple, and this technique is considered safe with viruses which establish a persistent infection. Increasing purification may lead to loss of immunogenicity however, and this often induces the necessity of coupling the purified peptides to an immunogenic carrier protein or adjuvant. Furthermore, the induced immunogenic response is restricted to selected antigens and vaccination may not provide significant protection against natural challenge.

DNA vaccines represent another recently developed method in use for vaccine trials. They usually comprise circular plasmids that include a gene encoding the target antigens. This gene is under the transcriptional control of a promoter region active in specific host cells, which then directly produce the foreign antigen. DNA vaccines offer greater control over the immunization process, because the investigator determines which antigens and co-stimulants to use, where to elicit the response, and whether to use immunostimulatory DNA sequences to modulate the type of immune response induced. In addition, DNA is relatively inexpensive and easier to produce than conventional vaccines. Safety issues with regard to DNA vaccines include risks of integration into cellular DNA and antibiotic resistance conferred through resistance genes present in the plasmid. However, many

preclinical trials with such vaccines have failed to show adverse reactions, and this method remains very promising for future vaccine studies.

The following sections describe different strategic approaches to protection against lentiviral infection, as well as encountered difficulties, in a series of relevant FIV vaccine studies carried out mainly in the last 10 years.

2.7.2.4.2. Conventional Vaccines:

The main concern in the use of inactivated whole virus or inactivated infected cell vaccines is the event of accidental infection caused by incomplete inactivation of the vaccine virus. Thus, such a vaccine approach is not feasible as vaccine against HIV. However, results from animal studies with such vaccines can provide new insights into the immune mechanisms of protection against lentiviruses. Conventional vaccines have indeed achieved the majority of success in experimental FIV vaccine trials against more severe challenge systems.

Several studies aimed at determining breadth of protection following immunization with a single strain inactivated whole virus vaccine. The first successful vaccine trial, reported in 1991, described efficient protection of a whole inactivated vaccine derived from FIV-PET, against an in vitro derived homologous strain {Yamamoto et al. 1993}. Further developing this study, {Hosie, et al. 1995} tested whether vaccination of cats with FIV-PET could induce protection against homologous (FIV-PET) or heterologous (FIV-GL8) challenge. Although both challenge virus strains belong to the FIV clade A, they present significant antigenic differences. As a consequence, vaccinated cats became infected following FIV-GL8 challenge. Later on however, the same group showed that inactivated FIV-PET vaccination significantly suppresses virus load and CD4+ T-cell loss in cats challenged with FIV-GL8 {Hosie, et al. 2000}. In contrast, inactivated FIV-M2, a clade B FIV, conferred protection only following mucosal, and not systemic challenge using a primary homologous virus isolate {Matteucci, et al. 1999}. Similarly, {Pu, et al. 2001} could not demonstrate efficient protection of cats vaccinated with whole inactivated clade D FIV (FIV-SHI) against homologous in vivo-derived challenge. These results suggest that protection against an in vivo-derived homologous strain inoculum is difficult to achieve with single-strain immunizations.

Similarly, single-subtype infected fixed cell vaccines seem to confer protection at best against homologous challenge strains. Various cell types have been tested over the years for their immunogenic potential as part of a vaccine. Infected cell vaccines derived from thymocyte or CrFK fibroblasts were not effective even against low dose, in vitro-derived homologous challenges, as shown by {Verschoor, et al. 1995}. Overall, successful cell-derived conventional vaccines have been developed from infected feline T-lymphocyte cell

lines. Greater than 90% protection was observed with a vaccine derived from chronically FIV-PET (subtype A) infected FL-4, an IL-2 independent feline T-cell line, against in vitro-derived homologous and slightly heterologous challenge inoculums {Yamamoto, et al. 1993}. In a similar study, Matteucci and colleagues (1996) demonstrated homologous protection of cats after vaccination with FIV-M2 (subtype B) infected MBM cells, an IL-2 dependent feline T lymphoblastoid cell line developed by the same group in 1995. A study experimenting immunization with autologous FIV-M2 infected lymphoblasts however failed to show efficient protection, even against a low dose of homologous ex vivo FIV challenge {Giannecchini, et al. 2002}.

The lack of heterologous protection of single subtype inactivated infected-cell or whole vaccines led to the multi-subtype vaccine approach, aiming at broadening immunity and protection. The first dual subtype FIV vaccine, consisting of inactivated subtype A- and D-infected cells, provided protection against in vitro-derived homologous strains but was unfortunately not tested against heterologous strains {Hohdatsu, et al. 1997}. An improved dual-subtype FIV vaccine, consisting of inactivated whole viruses of subtypes A and D, elicited strong anti-FIV cellular immunity and broad spectrum VN antibody activities In addition, it provided broadened protection for cats against homologous and heterologous challenges using in-vivo derived inoculum {Pu, et al. 2001}. This vaccine turned out to be the prototype of Fel-O-Vax FIV, a dual subtype vaccine produced with FIV subtypes A and D, and formulated with a proprietary adjuvant system, approved by the United States Department of Agriculture, and commercially released by Fort Dodge Animal health in 2002. The efficacy of this vaccine was demonstrated in a vaccination study designed to meet various requirements for registering the vaccine {Huang, et al. 2004}. Eight week old kittens were vaccinated with the vaccine, challenged 12 months later with a heterologous FIV strain, and monitored for FIV viremia. Only 16% of the cats developed viremia, while 90% of the controls became persistently infected with FIV, which demonstrated not only that the vaccine was efficacious and but also that immunity lasts for at least 12 months. The same study included a field safety trial in which 689 cats of various breeds, ages, and vaccination histories received a total of 2051 doses of the vaccine and thus demonstrated significant safety of the vaccine, as only 1% of the vaccinated cats presented mild reactions of short duration. Moreover, similarly to its prototype, which had demonstrated the potential to protect cats against heterologous challenge, Fel-O-Vax was shown to effectively protect cats against virulent challenge with a subtype B isolate, reported to be most common in the USA {Pu, et al. 2005}. A contact study, more closely reflecting field conditions, confirmed these findings: 6 vaccinated and 8 control cats housed with 5 challenge cats infected with a subtype B FIV strain were monitored for infection over

several years. All vaccinated animals remained protected whereas 50% of the unvaccinated cats became infected, suggesting the vaccine exhibits broad efficacy against genetically diverse FIV, even under contact challenge {Kusuhara, et al. 2005}. Unfortunately, Fel-O-Vax has a major conflict with current FIV diagnostics, including ELISA and Western Blot technologies. Indeed, this conventional inactivated FIV vaccine induces broad spectrum antibody production with long lasting titers to different FIV proteins. The precedent for using a whole virus vaccine in cats has been established with FeLV vaccine. No known cases of accidental infection due to improper inactivation of the virus have been reported with FeLV vaccines. With improved inactivation methods of vaccine virus and better adjuvant formulations, inactivated or killed vaccine approaches continue to be promising for future veterinary vaccine development.

2.7.2.4.3. Subunit Vaccines:

Recombinant subunit vaccine trials include purified viral proteins, synthetic peptide and live viral or bacterial vector-based vaccines. Unfortunately, although these products induce high rates of cellular responses accompanied by antibodies aimed at the specific targeted proteins, limited success has been obtained with this type of immunization against lentiviruses.

Many studies have based their trials on envelope antigens, as these are considered major targets of the immune system, and, as a consequence, appropriate components in vaccine development. The relative ease in production of satisfyingly pure products additionally renders recombinant envelope components especially attractive in use as immunogens for such studies. Furthermore, presence of anti-gag antibodies allows clear differentiation of seroconversion induced by natural infection from vaccine-induced humoral response. It was then quite disappointing, as a study using highly purified denatured or native recombinant SU proteins originally expressed in the Baculovirus system and in E. coli, could not demonstrate complete protection against homologous and heterologous challenge infection despite induction of a considerable humoral response {Lutz, et al. 1995}. In an effort to increase the immune response and thus the protection rate observed in this study, recombinant SU proteins were further tested in a series of immunization trials assessing feasibility of combining these peptides with either QS-21 adjuvant or Freund’s adjuvant together with the recombinant nucleocapsid protein (protein NC) of rabies virus. Better results in this experiment indicated that conjunction of recombinant FIV SU with appropriate adjuvants leads to partial protection against FIV challenge infection {Leutenegger, et al. 1998}. With similar objectives, {Hosie, et al. 1996} tested immunoaffinity purified gp120 for its prophylactic potential in FIV infection. Again, although immunized cats presented a lower viral load in PBMC than control cats, this vaccine failed

to confer complete protection following challenge. A more recent study, aiming determination of the level of crossreactivity between HIV-1 and FIV, demonstrated an overall protection rate of 78% against FIV challenge infection using in vivo derived inocula of both subtype B and recombinant subtype A/B, in cats immunized with adjuvanted recombinant HIV-1 p24 {Coleman, et al. 2005}. A vaccine consisting of only HIV-1 p24 protein thus appears to be efficacious against FIV infection, suggesting that the epitopes shared by members of the lentivirus family may have protective properties. Interestingly, such protection did not correlate with antibody titers, and Th1- promoting cytokines, supplemented in the adjuvant, greatly enhanced the HIV-1 p24 vaccine efficacy. These findings indicate that cross-protection observed in HIV-1 p24 vaccinated cats was most likely mediated by the cellular immunity. Future studies should determine whether the FIV-cat model using HIV-1 vaccine is a useful small animal AIDS model to characterize protective epitopes for HIV-1 vaccine design.

Another approach to development of subunit vaccines was the production of synthetic peptides. Again, most studies have based their immunization trials on envelope antigens, with limited success. Thus, immunization with a multiple antigenic peptide containing the synthetic prototype of an immunodominant neutralization epitope located within the V3 domain of the FIV envelope glycoprotein failed to confer protection in all of the immunized cats, although the peptide induced both specific cell-mediated and humoral immune responses {Flynn, et al. 1995}. In the same way, cats immunized with a 46-residue multiepitopic synthetic peptide of FIV comprising immunodominant epitopes present in V3 of the envelope glycoprotein, TM glycoprotein, and p24 gag core protein, developed an elaborate humoral immune response, but were not protected against challenge infection {Flynn, et al. 1997}. As Lombardi and coworkers {1996} demonstrated a remarkable and specific antiviral effect against the homologous and heterologous isolates using synthetic peptides representing either a conserved region of the SU protein or a hypervariable region of the TM protein.

Live recombinant vector vaccines are able to present the desired viral proteins in native conformation more efficiently than the purified subunit vaccines, and thereby elicit an immunity that may be more effective. The choice of a vector system that optimally expresses the desired proteins remains however challenging. Similar to the purified and synthesized subunit vaccines, envelope amino acids remain the protective epitopes of choice. A recombinant replication-defective vaccine composed of adenovirus type 5 vector expressing FIV *env* gene, was found to be ineffective at inducing *env*-specific antibody responses and at protecting cats against homologous infection {Gonin, et al. 1995}. Disappointing results were also reported using a FIV mutant expressing the FIV *env* gene.

Despite several booster immunizations, FIV-specific antibody responses in vaccinated cats were only weak, and the vaccinates did not withstand challenge with a low dose of homologous virus {Verschoor, et al. 1996}. Recently, {Burkhard, et al. 2002} and colleagues (2002) used Venezuelan equine encephalitis (VEE) virus-replicon particles (VRP) to generate FIV *gag*- and *env*-expressing vaccine vectors. All cats became infected following vaginal challenge with high dose, pathogenic cell-associated FIV-NCSU, although relative early maintenance of CD4+ cells was seen in FIV-immunized cats. The best results in vaccination against FIV with a vector based system came from a recombinant canarypoxvirus (ALVAC)-based vaccine {Tellier, et al. 1998}. In this study, 2 of 3 cats immunized with the ALVAC FIV recombinants were protected from homologous FIV challenge in the presence of FIV-specific cytotoxic T-lymphocyte responses, but in the absence of FIV-specific humoral responses. Moreover, all 3 cats immunized with the ALVAC-FIV recombinant and boosted with FIV-infected cell vaccine were protected from homologous FIV challenge in the presence of both FIV-specific CTL and humoral responses. This vaccine combination also induced partial protection against a heterologous challenge, which took place 8 months after the initial challenge. In conclusion, ALVAC indicates more promising results than other vector systems for FIV vaccine studies, and immunization schemes employing ALVAC vector in combination with inactivated FIV-infected cell vaccine can generate protective immune responses that can cross-react with FIV isolates that are genetically distinct from the vaccine strains.

2.7.2.4.4. DNA Vaccines:

In the late 1990s, DNA-mediated immunization emerged as a promising alternative in the development of viral vaccines, with protective immunity against viral and non-viral pathogens. This method had mainly attracted interest in lentiviral research for its potential to elicit strong cellular activity. Various trials effectively succeeded in reduction of viral load and prevention of disease after challenge, however complete protection was not achieved in either the HIV-or SIV-infected non-human primate models or FIV-infected domestic cats. A common approach to DNA vaccination trials has been the generation of live attenuated viruses with replication-defective but full-length proviral genomes, which express both structural and regulatory proteins in vivo. Moreover, inclusion of cytokine genes into a DNA vaccine enables increase of its immunogenicity and allows induction of desired types of immune responses. In 1998, {Hosie, et al. 1998} and colleagues were the first to test DNA vaccination in the FIV model. They vaccinated cats with a FIV mutant containing an in-frame deletion in *pol* (FIVΔRT), adjuvanted or not with INF-γ DNA. Altogether, the immunization with FIVΔRT elicited cytotoxic T-cell responses to FIV *gag* and *env* in the absence of a serological response. After challenge with homologous virus, 4 of the

10 vaccinates remained seronegative and virus free, while the rest displayed significantly lower proviral and viral loads. Conjunction of INF-γ DNA in the vaccine increased the protection rate. Unfortunately, further studies failed to demonstrate protection potential of this FIV mutant against heterologous strains and more virulent challenges, thus excluding its possible utility in the field {Hosie, et al. 2000}. In the same way, FIV mutants containing an in-frame deletion in the integrase gene (FIVΔIN) conferred protection against homologous, but not heterologous challenge {Dunham, et al. 2002}.

FIV mutant containing deletions in the viral accessory gene *vif* (FIVΔvif) {Lockridge, et al. 2000} or in the *orf-A* gene (FIVΔorf-A) {Pistello, et al. 2005}, also indicated significant protection against homologous challenge. Again, these mutants induced mostly specific cellular responses in the host, although anti-*env* and anti-*gag* antibodies were detected in several cats. In both studies, no reversion to wild-type virus occurred in the vaccinated cats, indicating relative safety in this type of viral attenuation for use as vaccine. Unfortunately, efficacy against genetically diverse strains was not tested, thus not allowing definitive conclusions about their relevance in the field.

To increase efficiency in expression of the incorporated genes, other vaccine trials have experimented the gene gun bombardment as vaccine DNA delivery. Such inoculations occur intradermally, whereby DNA-coated gold beads are directly shot into the keratinocyte nucleus. Advantages of this method include requirement of only low amounts of DNA and constant efficiency of transfection, with relative independence of vaccine dose from the size of the animal. In 2000 Boretti and colleagues immunized cats by the ballistic transfer of gold particles coated with minimalistic, immunogenic defined gene expression (MIDGE) vectors coding for FIV surface and partial transmembrane proteins, complemented or not with feline IL-12 DNA. These vectors consist of the double stranded expression sequence flanked by a phosphorylated oligodeoxynucleotide hairpin sequence on both ends. MIDGE particles thus lack sequence elements present on conventional plasmid constructs that could compromise the safety and immunological efficacy of the vaccine. Interestingly, 3 out of 4 cats immunized with MIDGE containing FIV *env* coding particles together with IL-12 DNA were protected against homologous challenge. Indeed, only one cat in this group became provirus positive, displaying however a greatly reduced viral load. These findings suggest that FIV-DNA vaccines, in combination with feline IL-12 DNA, may provide a promising way of inducing a protective immune response against FIV infection.

2.7.2.5. Enhancement of viral replication after challenge infection:

Viruses initiate infection by attaching to host cells via interaction between viral surface proteins and specific receptor/co-receptor molecules on target cells. Antibodies specific for

the viral surface proteins often inhibit this step of the infection cycle, resulting in reduced infectivity, and inducing so-called virus neutralization. In some circumstances, however, such antibodies have been shown to potentate viral infection. This phenomenon is known as antibody-dependant enhancement (ADE). Different mechanisms of ADE have been thoroughly described for several virus-cell systems in vitro, however in vivo relevance of ADE remains unclear. Obviously, further characterization of ADE is of great interest in the understanding of the pathogenesis of diverse viral diseases.

Several studies have focused on the importance of ADE in retroviral infections. At least three different mechanisms for ADE of HIV infection in vitro have been hypothesized: **a)** Interaction between antibody and cellular Fc receptor (FcR), **b)** interaction between the cellular complement receptor (CR) and the complement factors, induced by activation of the classical complement pathway during viral infection, and **c)** antibody or soluble factor induced conformational change in viral envelope, thus activating the glycoprotein and facilitating membrane fusion. Interestingly, it seems that viral infection through ADE uses a more efficient intracellular viral replication pathway, which suppresses expression of antiviral genes such as tumour necrosis factor and inducible nitric oxide synthase. Unfortunately, clinical significance of all types of ADE in HIV infection remains very controversial {Fust, et al. 1997}.

In the case of FIV, both antibody-dependent and independent responses have been associated with viral replication enhancement, thus complicating the understanding of immune processes and the development of vaccines. In 1992, {Hosie, et al. 1992}vaccinated cats either with purified FIV incorporated into immune stimulating complexes (ISCOMs), recombinant FIV p24 ISCOMs, or a fixed, inactivated cell vaccine in Quil A adjuvant, and thereby observed a more rapid viremia in vaccinated cats compared to control cats. Elevated or low titers of anti-p24, anti-env and neutralizing antibodies in the vaccinated cats seemed to play no role in the outcome of the challenge. Instead, 100% of the vaccinated cats became viraemic compared with 78% of the controls. In the same way, vaccination with fixed autologous FIV-infected cells did not protect cats against challenge infection despite induction of FIV-specific humoral responses. Again, accelerated virus replication was observed {Karlas, et al. 1999}.

When analyzing such results, it is important to keep in mind that whether antibodies enhance or neutralize seems to depend on the balance between a numbers of factors, including virus strain and dose, host-cell antibody combination, and the concentration and epitope specificity of the antibody.

Further studies have focused on the importance of the retroviral envelope in enhancement, as it is known to be the principal target for viral neutralizing antibodies. Indeed, the majority

of promising HIV vaccines undergoing clinical trials have been based on the viral envelope. However, monoclonal antibodies directed against a conserved region of the HIV transmembrane glycoprotein, called the principal immunodominant domain (PID), were shown to induce enhancement of infection on several cell types, thus raising concern about the efficacy of such vaccines. In this sense, immunization of cats with recombinant vaccinia virus-expressed FIV envelope glycoprotein, either incorporated in ISCOMS or adjuvanted with Quil A, resulted in enhanced infectivity of FIV. Additionally, as the observed enhancement could be transferred to naive cats with plasma collected at the day of challenge, the authors proposed that enhancement was mediated by anti-*env* antibodies {Siebelink, et al. 1995}. With the intention to further develop this hypothesis, Richardson and coworkers {1997} undertook an evaluation of the effect of vaccination with the FIV *env* gene on the development of infection in cats after challenge. Three groups of cats were thereby immunized using plasmid DNAs expressing either the wild-type envelope or two envelopes bearing mutations in the PID of the transmembrane glycoprotein. This genetic immunization elicited low or undetectable levels of antibodies directed against envelope glycoproteins, hypothetically due to the vaccination protocol used. Upon homologous challenge, determination of plasma virus load showed that the acute phase of viral infection occurred earlier in all three groups of cats immunized with the FIV envelope than in the control cats. Although the influence of antibodies cannot be completely excluded, the authors found no evidence for the presence of antibodies or other soluble factors capable of augmenting viral infection in vitro and therefore speculate that other immune phenomena, such as cellular activation, may have caused the acceleration of infection. FIV is known to replicate more efficiently in activated cells both in vitro and in vivo, as has been shown for HIV-1, and can infect B and T lymphocytes. Specific priming of cells of T and B lineages by *env* vaccination, while insufficient for the induction of detectable levels of antibodies, may have been sufficient to render cells more susceptible to viral infection. This study opens discussion to mechanisms unrelated to enhancing antibodies linked with acceleration of virus replication.

A recent study focused on the influence of antigenic properties of live attenuated vaccines in their protection potential against lentivirus infection. Random amino acid substitutions were introduced into the transmembrane envelope glycoprotein of FIV, within the PID, which notably bears immunodominant B-cell epitopes. Amongst a wide set of mutants, mutations that modified antibody specificity without abolishing infectivity ex vivo were selected to infect a group of SPF cats. After 1 year of infection, the cats were superinfected with a heterologous strain of FIV. One selected mutant, designated TN92, thereby succeeded in providing a significant protection of the cats against high viral loads.

The authors suggest that this protection correlates with a decrease, in TN92, of the immunogenicity of a B-cell epitope potentially involved in antibody enhancement of infection, thus emphasizing the importance of PID in the induction of such antibodies {Broche-Pierre, et al. 2005}.

Viral enhancement warrants caution in the design of antiviral vaccines. Moreover, there should be considerable concern over the use of vaccines against viruses that induce infectivity enhancing antibodies, as these vaccines could then predispose the host to persistent infection or lead to selection of “enhancable” virus types. It has been shown that antibody responses induced by subunit vaccines tend to be associated with severe disease by several retroviruses. A possible approach to the development of such vaccines against this virus family might be the induction of cellular immunity rather than antibodies. In this way, plasmid DNA and viral vector-based vaccines, as well as live attenuated vaccines, can be designed to induce strong cytotoxic T-lymphocyte responses that avoid harmful antibody responses.

2.8. Cytokine and antiviral effect:

Cytokines are soluble proteinaceous substances produced by a wide variety of haemopoietic and non-haemopoietic cell types, and are critical to the functioning of both innate and adaptive immune responses. Apart from their role in the development and functioning of the immune system, and their aberrant modes of secretion in a variety of immunological, inflammatory and infectious diseases, cytokines are also involved in several developmental processes during human embryogenesis. The effects of cytokines are mediated by their binding to a specific cell-surface receptor and the subsequent initiation of various intracellular signalling cascades that produce a wide variety of effects on the functioning of the cell. This may include the upregulation and/or downregulation of several genes and their transcription factors, that result in production of other cytokines, or increase in the number of surface receptors for other molecules, or suppress their own effect by feedback inhibition.

Therefore, cytokines are characterised by considerable redundancy, in that many cytokines can share similar functions. In a similar manner, cytokines are also pleotropic, in that they are capable of acting on many different cell types. Of course, this would be an anticipated corollary if one considers the simple fact that a given cell type may express receptors for more than one cytokine, or that many different tissues can express receptors for the same cytokine.

Generalisation of functions is not possible with cytokines; nonetheless, their actions may be comfortably grouped as:

* Autocrine, if the cytokine acts on the cell that secretes it.

* Paracrine, if the action is restricted to the immediate vicinity of a cytokine's secretion.

* Endocrine, if the cytokine diffuses to distant regions of the body (carried by blood or plasma) and mediates its effects on different tissues.

Cytokines have been variously named as lymphokines, interleukins and chemokines, based on their presumed function, and their cell of secretion or target of action. In view of the fact that cytokines are characterised by considerable redundancy and pleotropism, such a distinction has, with few exceptions, become largely obsolete.

The term interleukin was initially used by researchers for those cytokines whose presumed targets are principally leukocytes. The term chemokine referred to a specific class of cytokines that mediated chemoattraction (chemotaxis) between cells. The latter term alone has been retained (see below); interleukins are now used largely for designation of newer cytokine molecules discovered every day, and have little significance attached to their presumed function.

Of note, IL-8 (interleukin-8) is the only chemokine originally named an interleukin.

Classification:

Structural homology has been able to partially distinguish between cytokines that do not demonstrate a considerable degree of redundancy so that they can be classified into four types:

* The four α-helix bundle family-member cytokines have three-dimensional structures with four bundles of α-helices. This family in turn is divided into three sub-families:

1. The IL-2 subfamily
2. The interferon (IFN) subfamily
3. The IL-10 subfamily.

The first of these three subfamilies is the largest. It contains several non-immunological cytokines including erythropoietin (EPO) and thrombopoietin (THPO). Also, four α-helix bundle cytokines can be grouped into *long-chain* and *short-chain* cytokines.

* The IL-1 family, which primarily includes IL-1 and IL-18

* The IL-17 family, which is yet not completely characterized, though member cytokines have a specific effect in promoting proliferation of T-cells that cause cytotoxic effects {Snyderman, et al. 1999}.

Chemokines:

A more clinically and experimentally useful classification divides immunological cytokines into those that promote the proliferation and functioning of helper T-cells type 1 (example, IL-1, IFN-γ etc.) and helper T-cells type 2 (IL-4, IL-10, IL-13, TGF-β etc.), respectively. It is remarkable that the cytokines that belong to one of these sub-sets tend to inhibit the

effects of their counterparts - a tendency under intensive study for their possible role in the pathogenesis of autoimmune disorders.

Cytokine Receptors:

In recent years, the cytokine receptors have come to demand the attention of several investigators, partly because of their remarkable characteristics, and partly because a deficiency of cytokine receptors have now been directly linked to certain debilitating immunodeficiency states. In this regard, and also because the redundancy and pleomorphism of cytokines are in fact a consequence of their homologous receptors, many authorities are now of the opinion that a classification of cytokine receptors would be more clinically and experimentally useful.

A classification of cytokine receptors based on their three-dimensional structure has therefore been attempted.

*** Immunoglobulin (Ig) superfamily**, which are ubiquitously present throughout several cells and tissues of the vertebrate body, and share structural homology with immunoglobulins (antibodies), cell-adhesion molecules, and even some cytokines. Examples: IL-1 receptor types.

*** Haemopoietic Growth Factor (type 1) family**, whose members have certain conserved motifs in their extracellular amino-acid domain. The IL-2 receptor belongs to this chain, whose γ-chain deficiency is directly responsible for X-linked form of Severe Combined Immunodeficiency (X-SCID).

*** Interferon (type 2) family** whose members are receptors for IFN β and γ.

*** Tumour Necrosis Factor (TNF) (type 3) family** whose members share a cysteine-rich common extracellular binding domain, and includes several other non-cytokine ligands like CD40, CD27 and CD 30, besides the ligands on which the family is named (TNF).

***Seven transmembrane helix family,** the ubiquitous receptor type in the animal kingdom. All G-protein coupled receptors (for hormones and neurotransmitters) belong to this family. It is important to note that the chemokine receptors, two of which acting as binding proteins for the HIV virus (CXCR 4 and CCR 5), also belong to this family {Janeway, et al. 1999}.

2.8.1. Interleukin 10; IL10

2.8.1.1. Introduction

IL10 is a regulatory cytokine produced by antigen-presenting cells stimulated by bacterial pathogens, as well as human T cells of both Th1 and Th2 phenotype. IL-10 is the only cytokine that is known to exert a differential effect on IL-4-dependent isotype switching to IgE and IgG4 {Osugi, et al. 1997}.

Indeed, IL-10 has been shown to suppress IgE synthesis while enhancing IgG4 expression. Peripheral tolerance has been shown to be critically mediated by IL-10. Single nucleotide polymorphisms in the IL-10 gene have been associated with dysregulated IgE synthesis.

IL-10 has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. This cytokine can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway. In addition to these more systemic functions, knockout studies in mice also suggested the function of this cytokine as an essential immunoregulator in the intestinal tract. Immune responses are specific for both the antigen against which they are mounted and the class of response that is induced. For example, humoral (antibody-mediated) and delayed-type hypersensitivity (DTH) responses can be mutually exclusive. Tuberculoid leprosy is accompanied by a strong DTH response that ultimately kills and clears the bacilli, while in lepromatous leprosy, with weak cell-mediated immunity, the organisms multiply and the disease persists. Vieira and colleagues demonstrated the existence of human cytokine synthesis inhibitory factor, which was called interleukin-10 {Vieira, et al. 1991}.

2.8.1.2. Function:

Gesser et al. {1997} Identified 2 functional domains of IL10 exerting different IL10-like activities, an observation that suggested that relatively small segments of these signal proteins are responsible for particular biologic functions.

Pinderski et al. {1999} found that IL10 blocks atherosclerotic events in vitro and in vivo. Terkeltaub {1999} suggested that IL10 may arrest and reverse the chronic inflammatory response in established atherosclerosis.

Franchimont et al. {1999} examined the ability of tumor necrosis factor-alpha (TNFα) and IL10 to differentially regulate the sensitivity of human monocytes/macrophages to glucocorticoids. Dexamethasone had different effects on lipopolysaccharide-induced TNF and IL10 secretion; whereas it suppressed TNFα in a dose-dependent fashion, its effect on IL10 secretion was biphasic, producing stimulation at lower doses and inhibition at higher doses. Lipopolysaccharide influenced the effect of dexamethasone on IL10 secretion. Pretreatment with TNFα diminished, and with IL10 improved, the ability of dexamethasone to suppress IL6 secretion in whole-blood cell cultures and to enhance IL1 receptor antagonist (IL1RN) secretion by U937 cells. TNFα decreased, while IL10 increased, the concentration of dexamethasone binding sites in these cells, with no discernible effect on their binding affinity. The authors concluded that glucocorticoids

differentially modulate TNFα and IL10 secretion by human monocytes in an lipopolysaccharide dose-dependent fashion, and that the sensitivity of these cells to glucocorticoids is altered by TNFα or IL10 pretreatment; TNFα blocks their effects, whereas IL10 acts synergistically with glucocorticoids.

Esposito et al. {2003} tested the hypothesis that low serum IL10 concentrations associate with the metabolic syndrome in obese women. Compared with 50 matched nonobese women, the prevalence of the metabolic syndrome (3 or more of the following abnormalities: waist circumference greater than 88 cm; triglycerides greater than 1.69 mmol/liter; high density lipoprotein cholesterol less than 1.29 mmol/liter; blood pressure greater than 130/85 mm Hg; glucose greater than 6.1 mmol/liter) was higher in 50 obese women (52% vs 16%; P less than 0.01). As a group, obese women had higher circulating levels of IL6, C-reactive protein, and IL10 than nonobese women. In both obese and nonobese women, IL10 levels were lower in those with than in women without the metabolic syndrome. These results showed that circulating levels of the antiinflammatory cytokine IL10 are elevated in obese women and that low IL10 levels are associated with the metabolic syndrome.

Ma et al. {2005} analyzed B-cell development in 14 patients with X-linked lymphoproliferative syndrome (XLP) and identified an extrinsic block in differentiation that was improved by the provision of exogenous IL10 or by ectopic expression of SH2D1A, which increased IL10 production by T cells. Ma et al. {2005} suggested that insufficient IL10 production may contribute to hypogammaglobulinemia in XLP.

Gene structure:

Kim et al. {1992} showed that the mouse IL10 gene contains 5 exons and spans about 5.2 kb of genomic DNA.

Mapping:

Kim et al. {1992} mapped the IL10 gene to mouse chromosome 1 by interspecific backcross analysis and to human chromosome 1 by PCR analysis of DNAs from a panel of hamster-human somatic cell hybrids.

Eskdale et al. {1997} mapped the IL10 gene to the junction between 1q31 and 1q32. For this they used 2 dinucleotide repeats that lie in the 5-prime flanking region of the IL10 gene to analyze its position on the Whitehead Institute Radiation Hybrid map of human chromosome 1. The theory of radiation hybrid mapping was reviewed by Cox et al. {1990}. The human genome is fragmented by irradiation, with the degree of fragmentation being dependent on the radiation dose. Chromosomal fragments are then randomly inserted into hamster cell lines where they often become integrated into the hamster genome, thereby creating radiation hybrids. Examining a panel of such hybrids by PCR generates a pattern

of positive and negative hybrid clones. The distance between test markers and known standards is given in centi-Rays (cR).

2.8.1.3. Molecular genetics:

Stimulation of human blood cultures with bacterial lipopolysaccharide (LPS) showed large interindividual variation in IL10 secretion, which has been shown to have a genetic component of over 70% {Westendorp, et al. 1997}. Eskdale et al. {1998} undertook to determine the extent to which the structure of the IL10 promoter contributed to the genetic variation in LPS-induced secretion of IL10. Turner et al. {1997} demonstrated a difference in IL10 secretion, in association with the presence or absence of an 'A' at position -1082 of the human IL10 promoter, following concanavalin-A stimulation of peripheral blood mononuclear cells. Eskdale et al. {1998} defined alleles at 2 microsatellite loci in the 4 kb immediately upstream of the human IL10 transcription initiation site in 132 individuals from 56 Dutch families and assigned the alleles as haplotypes. LPS-induced IL10 secretion was measured by ELISA and related to the IL10 promoter haplotypes present in 78 unrelated individuals from these families. Analysis showed that LPS-induced IL10 secretion from unrelated individuals varied with IL10 promoter haplotypes (P = 0.024). Those haplotypes containing the allele IL10.R3 were associated with lower IL10 secretion than haplotypes containing any other IL10.R allele; the haplotype IL10.R2/IL10.G14 was associated with highest IL10 secretion overall, whereas the haplotype IL10.R3/IL10.G7 was associated with lowest IL10 secretion. Monocytes are the main source of IL10, although many cell types can be stimulated to IL10 secretion. The results indicate that genetic heterogeneity not only controls whether antigen responsiveness occurs (via the major histocompatibility complex) but also, through cytokines and their receptors, the extent and direction in which that response may develop once antigen recognition has occurred.

Grove et al. {2000} examined the potential role for polymorphism in the IL10 gene in the pathogenesis of alcoholic liver disease. The allele frequencies for 2 single basepair substitutions at positions -627(C to A) and -1117(A to G) in the IL10 promoter region were determined in 287 heavy alcohol drinkers with biopsy-proven advanced liver disease, 107 heavy drinkers with no evidence of liver disease, and 227 healthy volunteers. Fifty percent of patients with alcoholic liver disease possessed the A allele at position -627, compared with 33% of normal individuals (p less than 0.0001) and 34% of drinkers with no or mild disease (p = 0.017). No significant difference in allele frequencies was noted at position -1117. The authors concluded that the -627A allele is associated with an increased risk of advanced liver disease in alcohol abusers. This appeared to be consistent with reports elsewhere that the -627A allele is associated with low levels of IL10 expression, a situation which favors inflammatory, immune-mediated, and profibrotic mechanisms of tissue injury.

High IL10 production is associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE). In addition, IL10 production levels are concordant in monozygotic twins {Westendorp et al., 1997}. The IL10 promoter is polymorphic and may account for different levels of cytokine production. Gibson et al. {2001} identified 7 novel SNPs in the distal region of the IL10 promoter and found that certain haplotypes are significantly associated with high or low IL10 production and with SLE in African Americans.

Using short tandem repeat polymorphism (i.e., microsatellite) analysis, Shin et al. {2000} identified significant genotype associations for human immunodeficiency virus-1 (HIV-1) infection and progression to acquired immunodeficiency syndrome (AIDS) with markers adjacent to and tracking common single nucleotide polymorphic variants in the IL10 promoter region. Individuals carrying the 5-prime -592A promoter allele were at increased risk for HIV-1 infection, and once infected they progressed to AIDS more rapidly than homozygotes for the alternative -592C/C genotype. Approximately 25 to 30% of long-term nonprogressors (i.e., those who avoid clinical AIDS for 10 or more years after HIV-1 infection) carried the -592C/C promoter genotype. Additional protection or susceptibility was associated with the relevant CCR5 and CCR2 alleles. Electrophoretic Mobility Shift Assay (EMSA) analysis indicated that the -592A allele, but not the 592C/C allele, retains a binding site for Enviromental Tobacco Smoke (ETS) family DNA-binding proteins, whereas both alleles interact with SP1. Shin et al. {2000} noted studies (e.g., Rosenwasser and Borish {1997} that showed that the -592A allele is associated with diminished IL10 production. They suggested that progression to AIDS might be retarded by immunotherapeutic strategies mimicking or enhancing the natural inhibitory role of IL10).

After organ transplantation, susceptibility to cancer is multifactorial, especially for skin carcinomas. Risk factors may include genetic susceptibilities, such as the control of cytokine production. IL10 is a cytokine that is implicated in tumorigenesis, and polymorphisms in its gene promoter correlate with differential amounts of production. Alamartine et al. {2003} investigated a possible association between IL10 gene promoter polymorphisms and the occurrence of skin carcinomas after renal transplantation. Seventy kidney transplant recipients who developed a squamous cell carcinoma or a basal cell carcinoma were examined for polymorphisms in the IL10 gene promoter using PCR-based methods. Single-basepair mutations were studied at positions -1082, -819, and -592. These patients were compared to 70 healthy controls and to 70 matched renal transplant recipients without cancer. The IL10 secretion capability was tested in a subgroup of 40 of these patients by in vitro stimulation of peripheral mononuclear cells. IL10 genotypes and haplotypes were differently distributed in kidney transplant recipients who developed a skin

carcinoma, but especially a squamous cell carcinoma, with an increased frequency of the GCC haplotype and a decreased frequency of the ATA haplotype. Alamartine et al. {2003} found a shift in the predicted phenotypes from the low production phenotype to the high production phenotype. Secretion of IL10 was strongly correlated to the production predicted phenotype, and tended to be higher in patients who developed a squamous cell carcinoma than in the others. These results indicated that IL10 gene polymorphisms and IL10 production capability may contribute to the development of skin squamous cell carcinomas after renal transplantation.

IL10 is thought to play a key role in psoriasis. Its highly polymorphic promoter contains 2 informative microsatellites, IL10.G and IL10.R. Asadullah et al. {2001} analyzed IL10 promoter polymorphisms in 78 patients and 80 healthy controls. The distribution of IL10.G and IL10.R microsatellite alleles did not vary between patients and controls. In addition, when the psoriasis patients were stratified according to age of onset (younger than 40, or 40 and older), no difference in allele distribution was observed; however, a clear differential distribution was revealed at the IL10.G locus when patients were stratified according to whether they had a positive family history of psoriasis ($p = 0.04$). This difference was due to an overrepresentation of the IL10.G13 allele in those patients with familial disease (40.4% vs 19.6%, chi square = 7.292, $p = 0.007$). The positive association of allele IL10.G13 with familial psoriasis was especially strong when patients with early onset were compared with those with early onset against a nonfamilial background (39.6% vs 14.5%, chi square = 8.959, $p = 0.003$). Patients with age of onset of less than 40 were 4-fold more likely to have a psoriatic family background if they carried the IL10.G13 allele. These data suggested that the IL10 locus contributes to the heritability of psoriasis susceptibility.

By means of genetic association analysis, Shin et al. {2003} showed that the IL10 haplotype IL10-ht2 was strongly associated with hepatocellular carcinoma in a well-characterized hepatitis B virus (HBV) cohort. The frequency of susceptible IL10-ht2 was much higher in HCC patients and significantly increased in order of susceptibility to HBV progression from chronic hepatitis to liver cirrhosis and HCC among hepatitis B patients. In addition, survival analysis clearly showed that the onset age of HCC was also accelerated among chronic hepatitis B patients who were carrying IL10-ht2. Shin et al. {2003} suggested that increased IL10 production mediated by IL10-ht2 accelerates progression of chronic HBV infection, especially to HCC development.

Summers et al. {2003} hypothesized that higher or lower production of IL10 could affect the production of inflammatory cytokines associated with sudden infant death syndrome (SIDS**)**. The IL10 promoter SNPs -1082A, -819T, and -592A define the low IL10 producer

haplotype. Summers et al. {2000} found that the ATA haplotype, and specifically the -592A allele, were significantly more frequent in a group of 23 SIDS cases compared with controls. There was no association between SIDS and a TNF promoter polymorphism or a TGFB1 coding region polymorphism. Summers et al. {2000} noted that the -592 allele is associated with IL10 production by monocytes and macrophages. They proposed that a deficit in IL10 may contribute to SIDS either by a tardy initiation of protective antibody production or by a lower capacity to inhibit inflammatory cytokine production.

In a larger study than that of Summers et al. {2000}, Opdal et al. {2003} found that the ATA haplotype promotot of IL10 was associated with sudden unexpected infant death due to infection. However, they found no association between the SNPs that define the ATA haplotype and SIDS.

Centenarians escape, or at least delay, age-associated diseases that normally cause mortality at earlier ages. Considerable evidence supports involvement of genetic components to longevity.

The major trait of the offspring of centenarians is a significantly reduced prevalence of cardiovascular diseases. Patients with atherosclerosis have a proinflammatory genotype. Gene polymorphisms for proinflammatory cytokines seem to contribute significantly to the risk of coronary heart disease. In 2 populations from north and south Italy, Lio et al. {2004} found a significantly higher frequency of the IL10 -1082G/G genotype (which is associated with increased production of IL10) among oldest participants than in controls and patients with acute myocardial infarction. Conversely, the frequency of the -1082A/A genotype, associated with low production of IL10, was significantly higher in patients with acute myocardial infarction than in controls and oldest old participants. Thus, high production of IL10 was protective for acute myocardial infarction and a determinative parameter for longevity. A genetic background protective against cardiovascular disease appeared to be a component of longevity. Lio et al. {2004} reasoned that since the human immune system evolved to control pathogens, proinflammatory responses were likely to be programmed by evolution to resist fatal infections; they also noted that low production of interleukin-10 is associated with an increased resistance to pathogens. Increased concentration of interleukin-10, however, might better control inflammatory responses induced by chronic vessel damage and reduce the risk of atherogenic complications. Lio et al. {2004} concluded that these conditions might result in an increased chance of long life in an environment with a reduced load of antigens (i.e., pathogens).

In a case-control study of 304 Australian patients with Crohn disease and 231 healthy controls, Fowler et al. (2005) found a significant association of the higher-producing IL10 -

1082G and TNF-alpha -857C alleles with disease. The association was strongest when these alleles were combined and persisted after multivariate analysis.

2.8.1.4. In vivo studies:

Keratinocytes have been demonstrated to be suitable target cells for gene therapy. Keratinocyte gene therapy may be appropriate for treating diseases caused by genetic defects that result in keratinocytes with abnormal proteins. Keratinocytes are relatively easy to obtain and can easily be monitored by the expression of transgenes. Keratinocytes can be used as bioreactors releasing the transgenic protein into the circulation, and the endocrine and systemic effects of the protein required for therapy can be monitored. IL10 plays a major role in suppressing immune and inflammatory responses by inhibiting the production of proinflammatory cytokines. Meng et al. {1998} examined the systemic effects of IL10 released from transduced keratinocytes. An expression vector was constructed for human IL10 and was injected into the dorsal skin of hairless rats. Local expression of IL10 mRNA and protein was detected by RT-PCR and immunohistochemical staining, respectively. Enzyme-linked immunosorbent assay showed that the amount of IL10 in the local keratinocytes and in the circulation increased with the dose of vector transferred. To determine whether circulating IL10 could inhibit the effector phase of contact hypersensitivity at a distant area of the skin, various doses of the vector were injected into the dorsal skin of sensitized rats before challenge on the ears. The results showed that the degree of swelling of the ears of treated rats was significantly lower than that in the negative control animals. These results suggested that the IL10 released from transduced keratinocytes can enter the bloodstream and cause biologic effects at areas distant of the skin. It may therefore be possible to treat systemic disease such as hemophilia B by use of keratinocyte gene therapy.

Targeted mutations in a variety of mouse genes produce colitis. Mice homozygous for a disrupted interleukin-10 gene {Kuhn et al., 1993} supported the hypothesis that a dysregulated immune response to enteric flora can trigger inflammatory bowel disease. The severity of the colitis depends on the inbred strain background in which the disrupted gene is placed. Colitic lesions are much more severe in C3H mice than in B6 mice. Farmer et al. {2001} identified modifiers of cytokine deficiency-induced colitis susceptibility (Cdcs) by using quantitative trait locus (QTL) analysis. A major C3H-derived colitogenic QTL on mouse chromosome 3 contributed to lesions in both cecum and colon, as well as colitis-related phenotypes such as spleen/body weight ratio, mesenteric lymph node/body weight ratio, and secretory IgA levels. Evidence for other C3H QTLs on chromosomes 1 and 2 was obtained. The resistant B6 background also contributed colitogenic QTLs.

Treatment with the immunoregulatory cytokines IL4 or IL10 can inhibit the development of type I diabetes mellitus in nonobese diabetic (NOD) mice, a model of the human disease. Such treatment can also inhibit the recurrence of disease, whether alloimmune and/or autoimmune, in mice receiving islet transplants. Goudy et al. {2001} tested the feasibility of muscle-directed gene therapy to prevent autoimmune diabetes in NOD mice. They developed recombinant adeno-associated virus (rAAV) vectors containing murine cDNAs for immunomodulatory cytokines IL4 or IL10. Skeletal muscle transduction of female NOD mice with IL10, but not IL4, completely abrogated diabetes. Recombinant AAV-IL10 transduction attenuated the production of insulin autoantibodies, quantitatively reduced pancreatic insulitis, maintained islet insulin content, and altered splenocyte cytokine responses to mitogenic stimulation. These results indicated the utility for rAAV, a vector with advantages for therapeutic gene delivery, to transfer immunoregulatory cytokines capable of preventing type I diabetes. In addition, these studies provided foundational support for the concept of using immunoregulatory agents delivered by rAAV to modulate a variety of disorders associated with deleterious immune responses, including allergic reactions, transplantation rejection, immunodeficiencies, and autoimmune disorders.
Lee et al. {2002} generated transgenic mice overexpressing IL10 in lung. In these mice, IL10 inhibited TNF production and neutrophil accumulation, induced mucus metaplasia, B- and T-cell-rich inflammation, and subepithelial fibrosis, and augmented expression of Gob5 (CLCA1), mucins, and IL13 mRNA. In mice lacking IL13, IL4ra (IL4R, or Stat6, transgenic IL10 induced inflammation and fibrosis, but not mucus metaplasia. Lee et al. {2002} concluded that IL10 induces airway remodeling accompanied by mucus metaplasia and tissue inflammation through multiple IL13-dependent and -independent mechanisms.

2.8.1.5. Allelic variants (selected examples)

2.8.1.5.1. Susceptibility to Human immunodeficiency virus type 1:

Shin et al. {2000} reported an increased susceptibility to HIV-1 infection and more rapid progression to AIDS in patients with a C-to-A polymorphism at position -592 of the IL10 promoter. The -592A allele reduces IL10 transcription by a factor of 2 to 4. The authors found that -592A allele-specific synthetic oligonucleotides did not bind certain Enviromental tobacco smoke (ETS**)** family transcription factors, which recognize the wildtype IL10 allele sequence. Heterozygosity and homozygosity with respect to the -592A allele was associated with accelerated AIDS progression, probably owing to downregulation of the inhibitory IL10 cytokine.
In 993 transplant recipients, Lin et al.{2003} found that the IL10 -592A/A genotype, as compared with the C/C genotype, was associated with a decreased risk of acute graft-versus-host disease (GVHD) and death in remission. A haplotype analysis showed that the

-592A allele was a specific marker for a promoter haplotype, T-C-A-T-A, defined by 5 polymorphisms at positions -3575, -2763, -1082, -819, and -592, respectively. Among recipients of hematopoietic cells from an HLA-identical sib, the -592A allele was shown to be a marker of a favorable outcome after transplantation. Cooke and Ferrara {2003} commented on the usefulness of information on IL10 genotype in clinical practice.

2.8.1.5.2. Progression of rheumatoid arthritis:

Lard et al. {2003} compared allele frequencies of the promoter -2849A/G polymorphism of the IL10 gene in 283 patients with rheumatoid arthritis, 413 patients with other rheumatic diseases, and 1,220 healthy controls. The IL10 genotype was not associated with the incidence of RA, but instead correlated with disease progression, with a significantly higher rate of joint destruction at 2 years observed in patients with a -2849G allele. RA patients with the G allele, which is associated with high IL10 production, also had higher autoantibody titers at baseline.

2.8.2. Interleukin-12:

2.8.2.1. Introduction:

Interleukin (IL) 12 naturally produced by dendric cells macrophages and human B-lymphoblastoid cells in reponse to antigenic stimulation. IL-12 also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF), is a heterodimeric pleiotropic cytokine made up of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. The IL-12 p40 subunit is shared by IL-23, another heterodimeric cytokine that has biological activities similar to, as well as distinct from, IL-12. IL-12 is produced by macrophages and B cells and has been shown to have multiple effects on T cells and natural killer (NK) cells. While mouse IL-12 is active on human and mouse cells, human IL-12 is not active on mouse cells {Kaliński, et al. 1997}.

IL12 and IL23 show overlapping, but also some distinct, biological properties. Overexpression of IL12 p40 seems to be instrumental in a variety of Th1-mediated immunopathologies, including Crohn's disease. Interleukin 12 (IL-12) is an important regulatory cytokine that has a function central to the initiation and regulation of cellular immune responses. It has the capacity to regulate the differentiation of naive T cells into TH1 cells, which is crucial in determining resistance and the type of response that will be elicited in response to a particular pathogen. It stimulates the growth and function of T cells and alters the normal cycle of apoptotic cell death.

2.8.2.2. Structure of IL-12:

IL-12 is one of a large group of cytokines that folds into a bundle of four alpha-helices. It is a heterodimer of 70kDa that is composed of two disulfide-linked subunits, with a mass of 35kDa and 40kDa. These subunits are coded by different and seemingly unrelated genes

(Brandhuber et al., 1987). Only a single receptor chain has been identified for IL-12, labeled the IL-12Rbeta1 receptor. Its structure of about 100kDa in humans and mice is most homologous to the leukemia inhibitory factor (LIF) receptor {Chua, et al. 1995}. IL-12 p40 and p35 chains are encoded by two separate genes that bear no apparent homology. The gene encoding the p40 chain is mapped to chromosome 5q31-q33, a region that encodes many cytokines and cytokine receptors, and the gene encoding the p35 chain is located on chromosome 3p12-3q13.2. The two genes that encode the murine p40 and p35 counterpart chains contain 70 and 60% sequence homology, respectively, to the human genes {Xiaojing, et al. 1996}.

2.8.2.3. IL-12 Gene Expression:

There are three methods to detect the cellular expression of IL-12. They are: Northern blot, RNAse protection, and competitive/quantitative PCR. Polyclonal antibodies to the p40 and p35 chains can be produced to detect the presence of IL-12. The production of IL-12 requires an apparent coordinated expression of both p40 and p35 chains that makes the formation somewhat challenging. Additionally, the p40 homodimer has been shown to have an antagonistic role in the mouse {Gillessen S., et al.1995}. Jaklien et at. {2006} demonstrated that constitutive overexpression of p40 in lungs negatively influences IL-12-mediated leucocyte migration and protection against lung tuberculosis. This suggests a novel antagonistic role for p40 homodimers in regulating the chemotactic bioactivity of IL-12 after pulmonary mycobacterial infection.

Furthermore, the p35 chain of IL-12 has a more widespread expression throughout the individual and its mRNA was found in both brain and lung tissue, while the p40 chain was not detected in either of these areas. It is thought that the p35 chain may have a function within the body independent of its regulatory capacity through IL-12. Upon activation of phagocytic cells, accumulation of IL-12 is observed to be somewhat delayed in relation to the expression of other inflammatory cytokines, such as TNF-α and IL-1β, and then subsides after several hours. The activation of the p40 chain requires active protein synthesis, mainly at the initiation of the stimulus. The production of IFN-γ has a very powerful effect in enhancing the ability of phagocytic cells to produce IL-12 and IL-12 also enhances the production of IFN- γ, creating a positive reinforcement loop. Early induction by IL-12 or IFN-γ expression is key to the initiation of the innate immune response.

2.8.2.4. Functions:

IL-12 is involved in the differentiation of naive T cells into Th1 cells, which are important for resistance against pathogens. It is known as a T cell stimulating factor, which can stimulate the growth and function of T cells. It stimulates the production of IFN-γ and tumor necrosis factor α from T and natural killer (NK) cells, and reduces IL-4 mediated

suppression of IFN-γ. T cells which produce IL-12 have a coreceptor, CD30, which is associated with IL-12 activity.

IL-12 plays an important role in the actitvities of natural killer cells and T lymphocytes. IL-12 mediates enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes. There also seems to be a link between IL-2 and the signal transduction of IL-12 in NK cells. IL-2 stimulates the expression of two IL-12 receptors, β1 and β2, maintaining the expression of a critical protein involved in IL-12 signaling in NK cells. Enhanced functional response is demonstrated by IFN-γ production and killing of target cells.

IL-12 also has antiangiogenic activity, which means it can block the formation of new blood vessels. It does this by increasing production of interferon gamma, which in turn increases the production of inducible protein-10 (IP-10). IP-10 then mediates this anti-angiogenic effect. Because of its ability to induce immune responses and its anti-angiogenic activity, there has been an interest in testing IL-12 as a possible anti-cancer drug. However, it has not been shown to have substantial activity in the tumors tested to this date.

IL-12 binds to the IL-12 receptor, which is a heterodimeric.

2.8.2.5. Signal transduction:

IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by β1 and β2. β2 is considered to play a key role in IL-12 function, since it is found on activated T cells and is stimulated by cytokines that promote Th1 cells development and inhibited by those that promote Th2 cells development. Upon binding, β2 becomes tyrosine phosphorylated and provides binding sites for kinases, Tyk2 and Jak2. These are important in activating critical proteins such as STAT4 which are implicated in IL-12 signaling in T cells and NK cells. This pathway is known as the JAK-STAT pathway.

2.8.2.6. IL-12 and autoimmunity:

IL-12 is linked with autoimmunity. Administration of IL-12 to people suffering from autoimmune diseases was shown to worsen the autoimmune phenomena. This is believed to be due to its key role in induction of Th1 immune responses. In contrast, IL-12 gene knock-out in mice or a treatment of mice with IL-12 specific antibodies ameliorated the disease.

2.8.2.7. Medical Uses:

IL-12 has great potential as a vaccine adjuvant for promoting cell-mediated immunity and a TH1 cell response. Not only does immunization with IL-12 as adjuvant promote a long-term and stable TH1 response, it also enhances the primary TH1 response when given in conjunction with other adjuvants. However, O'Garra notes that repeated exposure to antigen and IL-12 is necessary to establish a stable TH1 response {O'Garra, et al. 1996}.

One of the first succeseful application of IL-12 as adjuvant in an outbred species was reported in a FIV-DNA vaccine in cats {Boretti et al. 2000}.

2.8.2.8. IL-12 knockout mice:

Cytokine responses were monitored for IL-12 knockout mice in vivo to further define the important role of the differentiation of naive T cells into TH1 cells. This differentiation helps create a balance between the cell-mediated and humoral immunity. In this experiment, the knockout mice were observed to be completely viable and fertile, and displayed no developmental abnormalities. However, on an immunological level, these mice were noticed to have a reduced capacity to cause a TH1 cell response and also a relative inability to produce IFN-γ in response to toxins engulfed by phagocytic cells {Magram, et al. 1996}.

2.8.2.9. Function of Co-receptors in the action of IL-12 in mice:

Activated T cells were injected into mice and the mice were tested for antibodies that might be responsible for the regulation of T-cell responsiveness to the binding of IL-12. CD2 was identified as one of these regulators along with its major ligand, CD58, which binds to its adhesion portion and effectively inhibits the response of the T cells to bound IL-12. However, this regulation does not affect the binding of IL-12 in any way. In fact, the presentation of adhesion molecules by an antigen presenting cell, a monocyte for example, illustrates how these APCs can regulate the response of T cells to a cytokine, without disturbing the cytokine binding interaction {Gollob and Ritz, 1996}.

2.8.2.10. Normal Function/Signal Transduction involving IL-12:

The induction of a cell-mediated immune response to a specific antigen is regulated, for the most part, by the release of cytokines. IL-12 is produced by macrophages, monocytes, dendritic cells, and B cells in response to bacterial products and intracellular parasites. The biological effects of the production of IL-12 are directed at T cells and NK cells. IL-12 is responsible primarily for the subsequent production of IFN-γ and tumor necrosis factor-alpha (TNF-α) from both NK cells and helper T cells. Researchers have concluded in recent experiments that because IL-12 is responsible for the production of IFN-γ, its immunological action must be directed primarily to those cells that are capable of producing IFN-γ. The cells that produce IFN-γ most often are those activated T cells that also have the coreceptor, CD30, present on their surface. Therefore, CD30+ T cells are a target of the actions of IL-12 {Alzona, et al. 1994}. IL-12 also stimulates the rate at which NK cells and helper T cells proliferate following antigen activation. In addition, the lytic capacities of both NK and helper T cells are increased by the presence of IL-12. IL-12 has the specialized function of leading naive CD4+ T cells to differentiate toward the TH1 cell type in order to prepare for the release of IFN-γ and for the development of the cell-

mediated immune response {Hsieh, et al. 1993}. IL-12 and IL-2 are both important cytokines in the regulation of a cell-mediated immune response, IL-2 being responsible for stimulating the growth and proliferation of T cells, while IL-12 stimulates the differentiation of the CD4+ T cells into TH1 cells. The sites of phosphorylation and activation of particular transcription factors during the signaling pathways of these two cytokines are not completely understood.

It`s important to note the function IL-12 has in the regulation of the production of antibody isotypes. The direct binding of IL-12 to B cells causes a long-term enhancement of antibody production; in addition to the isotype switching that is caused by the induction. IL-12 plays a central role in both the induction and magnitude of a primary Th1 response. A critical question in designing vaccines for diseases requiring Th1 immunity such as Mycobacterium tuberculosis and Leishmania major is the requirements to sustain memory/effector Th1 cells in vivo. This report examines the role of IL-12 and antigen in sustaining Th1 responses sufficient for protective immunity to L. major after vaccination with LACK protein (LP) plus rIL-12 and LACK DNA. It shows that, after initial vaccination with LP plus rIL-12, supplemental boosting with either LP or rIL-12 is necessary but not sufficient to fully sustain long-term Th1 immunity. Moreover, endogenous IL-12 is also shown to be required for the induction, maintenance, and effector phase of the Th1 response after LACK DNA vaccination. Finally, IL-12 is required to sustain Th1 cells and control parasite growth in susceptible and resistant strains of mice during primary and secondary infection. Taken together, these data show that IL-12 is essential to sustain a sufficient number of memory/effector Th1 cells generated in vivo to mediate long-term protection to an intracellular pathogen. Genetic vaccination using MIDGE-based constructed for the expression of the surface-transmembrane protein domain of the FIV env and feline IL-12 DNA led to protection against homologous virus challenge in three out of four vaccinated cats {Boretti, et al. 2000}.

2.8.3. Interferons:

2.8.3.1. Definition:

Interferons are cytokines produced by white blood cells, fibroblasts, or T-cells as part of an immune response to a viral infection or other immune triggers. The name of the proteins comes from their ability to interfere with the production of new virus particles. This family of cytokines, comprising the type I or viral IFNs (consists mainly of α, β, γ, and Ω). Interferons (IFNs) regulate a number of host responses, including innate and adaptive immunity against viruses, bacteria, parasites, and neoplastic cells. These responses are dependent on the expression of IFN-stimulated genes (ISGs). Given the diversities in

these responses and their kinetics, it is conceivable that a number of different factors are required for controlling them, a vast body of information on this mechanism is available {Bonjardim, et al. 2005}.

2.8.3.2. Types of interferon:

There are four types of interferons: alfa, beta, gamma and Ω. Alfa and beta interferons, which are grouped together as type I interferon, are produced by white blood cells and fibroblasts. Gamma interferon (or type II interferon) is manufactured by T-cells. Production occurs when the T-cells are activated such as during an infection.

The alfa and beta interferons share some biological activities, but also have activities that are distinct from one another. These similarities and differences reflect the common and different binding of the interferons to various targets on the surfaces of human cells.

An interferon designated as beta-1b enhances the activity of T-cells, while simultaneously reducing the production cytokines that operate in the inflammatory response to infection and injury. In addition, this interferon retards the exposure of antigens on the surface of cells (and so lessens the development of an immune response to the antigens), and retards the appearance of lymphocytes in the central nervous system.

The reduction of the immune response can lessen the damage to nerve cells in diseases such as multiple sclerosis. In this disease, the immune system is stimulated to react against the myelin sheath that surrounds the cells resulting in demyelination, Demyelination produces a malfunction in the transmission of impulses from nerve to nerve and from nerve to muscle.

In the late 1980s, a large clinical trial to evaluate treatment of multiple sclerosis conducted in the United States and Canada evaluated the influence of interferon beta-1b (Betaseron, marketed by Berlex) made in bacteria using genetic engineering technology. Specifically, the bacterium *Escherichia coli* contained a piece of genetic material (plasmid) coding for human beta interferon. A two-year double-blind study demonstrated that those people receiving the interferon had fewer reappearances of the symptoms, and fewer nerves in the brain were damaged.

Betaseron was approved in 1993 by the U.S. Food and Drug Administration for use by people affected with multiple sclerosis. Other products followed {Rebif, et al. 2002}.

In dogs recombinant canine IFN-γ was shown to be beneficial in the treatment of atopic canine dermatitis {Yasukawa, et al. 2010}.

2.8.3.3. The Role of Interferon in preventing viral replication:

Interferon refers collectively to a family of three proteins that non-specifically inhibit viral replication inside host cells. In response to infection by a virus, various cell types secrete interferon into the extracellular fluid. Interferon then binds to plasma membrane receptors

on nearby cells, whether they are infected or not. It also enters the circulation and reaches cells at far-removed sites. Thus those cells can synthesize interferon and provide it to cells that cannot.

2.8.3.4. Treatment and therapeutic effect:

From the time Isaacs and Lindenmann {1957} discovered INF up to present {Darnell et al., 1994}, the ability of INFs to protect against viral infection has been continuously reaffirmed. Different from type II INF (INF-γ), which is made primerlly T cells after induction of the adaptive immune responses, type I INF, a major component of the innate immune system, comprises of a family of closely related proteins (INF-α) and a single gene product (INF-β) induced early during infection that inhibit virus replication after binding to a common receptor (receptor for INF-α and INF-β, IFNAR) {Quellet,et al., 1994} coupled to a janus kinase (JAK). The resulting events are activation of STAT1 and STAT2 followed by the formation of interferon-stimulated gene factor 3 (SGF3) compex composed of STAT1, STAT2, and IFN regulatory factor 9, which promotes serial synthesis of selected proteins that inhibit viral replication {Aaronson an Horvath, 2002; Darnell et al., 1994}. De Mari el al. {2004} showed that rFeIFN Ω initially had statistically significant therapeutic effects on clinical sighns and later on survival of cats with clinical sighns associated with FeLV infection and FelV/FIV coinfection.

2.8.3.4.1. Therapeutic use of feline interferon Ω:

The clinical efficacy of a recombinant feline interferon, rFe IFN Ω was evaluated under field conditions in cats presented with clinical signs associated with FeLV infection and FeLV/FIV coinfection. In this multicentric, double-blind, placebo-controlled trial, 81 cats meeting the inclusion criteria were randomly placed into 2 groups and treated subcutaneously with rFelFN Ω (1MU/kg per day) or placebo once daily for 5 consecutive days in 3 series (day 0, 14, 60). The cats were monitored for up to 1 year for clinical signs and mortality. During the initial 4-month period, IFN Ω treated cats (n = 39) had significantly reduced clinical scores compared with placebo (n = 42), with all cats having received concomitant supportive therapies. Compared with the control, the IFN-treated group showed significantly lower rates of mortality: 39% versus 59% (1.7-fold higher risk of death for controls) at the 9-month time point and 47% versus 59% (1.4-fold higher risk of death for controls) at the 12-month time point. The IFN treatment was associated with minor but consistent improvement in abnormal hematologic parameters (red blood cell count, packed cell volume, and white blood cell count), apparently underlying the positive effects of IFN on clinical parameters. These data demonstrate that rFeI FN Ω initially has statistically significant therapeutic effects on clinical signs and later on survival of cats with clinical signs associated with FeLV infection and FeLV/FIV coinfection {de Mari K., et al.

2004}. Recombinant FeIFN Ω was also found to have a anti-tumor effect in feline and canine mammary tumors {Penzo, et al. 2009}. IFN Ω has also been used with some success in canine dermatology {Carlotti, et al. 2009}.

A total of 12 clinically ill cats previously diagnosed as feline infectious peritonitis (FIP) were treated with a combination of recombinant feline interferon and glucocorticoid. A complete remission (over 2 years) and a partial remission (2 to 5 months) were observed in four (33.3%) and four (33.3%) cases, respectively. Those that survived for more than 2 years were all older cats (6 to 16 years old) with the effusive form of FIP {Ishida, et al. 2004}. Unfortunateley, this study has absolutely no value as the diagnosis “FIP” was not supported by firm criteria such as histology therefore; therefore, the claims made by the auther are more than doubtful.

In another study, the antiviral activity of recombinant feline interferon-γ (rFeIFN-γ) against feline immunodeficiency virus (FIV) was investigated. A persistently FIV(Bangston)-infected feline T cell line (FeT-J/Bang) was treated with either rFeIFN-γ, rFeIFN-γ, or recombinant human IFN-α2 (rHuIFN-α2), and the culture fluids were tested for antiviral activity by reverse transcriptase (RT) assay. FeT-J/Bang cell cultures treated with rFeIFN-γ showed dose-dependent inhibition of RT activity. In contrast, rFeIFN-γ treatment had no antiviral effect on FIV replication but instead caused a statistically significant enhancement on day 9 of culture {Tanabe, et al. 2001}.

Antiviral activity of rFeIFN-γ was also tested on feline peripheral blood mononuclear cells (PBMC). PBMC cultures were inoculated with FIV (Bangston) and simultaneously treated with either rFeIFN-Ω, rFeIFN-γ, or rHuIFN-α2. FeIFN-γ had no effect on FIV replication, unlike the rFeIFN-Ω and rHuIFN-α2, which had strong anti-FIV effects. In another study, rFeIFN-γ treatment was initiated 3 days before FIωV (Bangston) infection, on the day of FIV (Bangston) infection, or 3 days post-FIV (Bangston) infection and then tested for antiviral activity. The time of initiating rFeIFN-γ treatment had no effect on the antiviral activity. Hence, these results suggest that unlike rHuIFN-α2 and rFeIFN-Ω, rFeIFN-γ has no inhibitory effect on FIV replication in PBMC but causes a slight enhancement in a feline T cell line {Tanabe, et al. 2001}.

Bumsuk, et al. {2005} reported that two dissimilar viruses employ a common maneuver to cause a profound immunosuppression. Measles virus (MV) and lymphocytic choriomeningitis virus (LCMV) interfere with dendritic cell (DC) development and expansion in vivo and in vitro. The underlying mechanism for this is through the generation of type I interferon (IFN) that acts via a signal transducer and activator of a transcription (STAT) 2-dependent, but STAT1-independent, pathway. Thus, viruses subvert the known antiviral effect of type I IFN through STAT2-specific signaling to benefit their survival.

These observations have implications for understanding and developing therapies to treat diseases caused by immunosuppression and/or persistent infections.

2.8.3.4.2. Recommended dosage:

Interferons have to be injected. They are not taken perorally as the proteins will be digested and not absorbed as intact molecules. For use in multiple sclerosis, interferon beta-1a is injected into the muscle (intramuscular injection), and beta-1b is injected just below the skin (subcutaneous injection). The injections are usually given every other day. The recommended dose for beta-1a and 1b is 0.03 mg and 0.25 mg, respectively. Initial doses of beta-1b should be far less (i.e., 0.0625 mg), with a gradual increase in dose over six weeks.

Precautions:

Patients who have had seizures or who are at risk for a seizure should be closely monitored following the injection of interferon, as should those with heart disorders such as angina pectoris, congestive heart failure, or an irregular heartbeat.

It is not known if interferon can be expressed in breast milk. Concerned mothers may opt to cease breast-feeding while receiving interferon therapy.

Side effects:

Interferon beta 1-a and 1-b commonly induce flu-like symptoms, including fever, chills, sweating, muscle aches, and tiredness. These side effects tend to diminish with time. Menstrual cycle changes have also been documented in a significant number of women. Far less commonly, interferon beta 1-a and 1-b can produce suicidal feelings in someone who is already clinically depressed. Death of cells around an injection site (necrosis) can occur, as can swelling and bruising. Allergic reactions are possible. The massive and sometimes fatal allergic reaction termed anaphylaxis occurs rarely. Other side effects include liver and thyroid malfunction, and altered blood chemistry (fewer platelets and red and white blood cells).

3. Material and Methods:

3.1. Animals:

20 male Specific Pathogen Free (SPF) domestic cats, 10 weeks old obtained from the cat colony maintained at Liberty Research, Inc. USA were housed under SPF conditions at the cat housing facility of the clinical laboratory. The animals were adapted for 8 weeks, assigned to 1 of 2 groups according to age, visually inspected daily and clinically examined once weekly. At the end of the experiment all cats were euthanized and samples were collected for further analysis.

3.2. Health of the cats:

Shortly after arrival of the animals, they were tested for absence of the most impotant feline pathogens FCV, FHV, FPV, FCoV, FeLV, and FIV as shown below.

3.2.1. Rectal swabs for FCoV and FPV:

Two days after arriving rectal swabs were collected and total nucleic acids were extracted from the swabs using the Mag NA Pure LC machine (Roche GmbH, Mannheim, Germany). Before the extraction was started, 200µl HBBS were added to each sample and the samples were Vortexed for 5 Seconds, incubated at 42°C for 10 minutes & centrifuged at 8000 rpm for 1 minute. The swabs were turned around in the ebendorf tube and were centrifuged again. The swabs were discarded and 300 µl of lysis buffer from TNA extraction kit were added to each sample for 10 minutes. Extraction was done according to the manufacturer's recommendation (MagNA Pure LC TNA Isolation Kit, Roche GmbH, Mannheim, Germany).

3.2.2. Oropharyngeal and Ocular swabs for FCV and FHV-1:

Two days after arriving oropharyngeal and ocular swabs were collected for extraction of total nucleic acid by the Mag NA Pure LC machine. Before extraction, 200 µl HBBS were added to each sample which were then vortexed for 5 Seconds. The samples were incubated at 42°C for 10 minutes & centrifuged 8000 rpm for 1 minute. The extracts were treated as mentioned above for rectal swabs.

3.2.3. EDTA blood for Haematology and the detection of FeLV and FIV:

One week after arriving we collected 1 ml EDTA blood of which 500 µl were used for Haematology. Three hundred µl lysis buffers were added to 200 µl of blood for TNA extraction using Mag NA Pure LC machine. Lysis was performed using the TNA external lysis program and TNA were extracted according to the manufacturer's recommendation (MagNA Pure LC TNA Isolation Kit, Roche GmbH, Mannheim, Germany).

3.2.3.1. Hematology:

The EDTA blood samples were analysed for red cells counts, haemoglobin, hematocrit, white cell counts and platelet count using the Cell Dyn 3500 instrument (Abbott AG, Baar, Switzerland). Due to the fact that in the cat the large platelets can not be separated from small erythrocytes, the values may not always be correct. The analysis was done to detect a passible decline or increase in concentration during observation period.

For the determination of the leukocyte population, blood smears were prepared, stained under routine conditions and differential counts were made by 2 laboratory technicians evaluating 100 cells each.

3.3. Treatment of the cats with Interferon and placebo:

The cats were assigned randomly to one of two groups which were then treated by the interferon preparation or a placebo. Care was taken that siblings were separated in order to minimize genetic influence and that the age of the cats was similar in both groups. As a consequence of the assignment, the weight was slightly different between the two groups. The two groups were treated by subcutaneous injection of the drug over a period of 5 days before infection. Injection of the drug with the dose of 0.1ml/kg adjusted to a concentration of $1X10^6$ U / kg per day.

3.4. Challenge virus:

Twenty four hours after the last injection the GL-8 virus stock was brought to the laboratory in dry ice, the virus stock was warmed rapidly in a water bath to reach 0°C and a 1:5000 dilution was made on wet ice, (using 1400 µl stock virus + 68.6 ml sterile RPMI 1640 medium with 10% FCS) to obtain a dose of 100 CID_{50} / ml per cat. Cats were inoculated intraperitoneally (i.p) with the FIV GL-8 virus which had been prepared in primary tissue culture.

3.5. Blood collection:

Blood samples were obtained weekly from all cats under light anaesthesia (Ketamin 20 mg/ kg bw and Midazolam 0.1mg / kg bw) starting on week 0 (time point of infection) till week 11, with the exception of week 10. Additionally, samples were collected twice during the prophylactic treatment, i.e. 5 days (w0-5d) and 3 days (w0-3d) before challenge infection. Two ml EDTA blood and 2 ml Heparin blood were collected weekly.

The EDTA blood samples were analysed weekly for reticulocytes, haemoglobin, hematocrit, leukocytes and platelet count using the Cell Dyn 3500 instrument (Abbott AG, Baar, Switzerland) see paragraph 3.2.3.1.

3.5.1. Proviral load detection in blood:

FIV proviral load were determined by quantitative TaqMan PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) under

conditions described. The analytical sensitivity of this PCR method, in which a 133 base pair long stretch of the *gag* gene is amplified was determined to be one copy of provirus within 1 µg genomic DNA {Leutenegger, et al. 1999}.

3.5.2. Viral load detection in plasma:

Plasma was collected by centrifugation of the remaining EDTA blood at 3500 rpm for 10 minutes and double samples of 200 µl plasma were stored in -80°C immediately for subsequent TNA extraction. Late, TNA was extracted according to the manufacturer's recommendation (MagNA Pure LC TNA Isolation Kit, Roche GmbH, Mannheim, Germany). Plasma viral RNA loads were measured at regular intervals throughout the experimental period to evaluate viral load changes in cats using an ABI Prism 7700 sequence Detection System under conditions described.

3.5.3. Detection of Antibodies to TM by ELISA:

Antibodies against a recombinant FIV transmembrane antigen were measured with an enzyme-liked immunosorbent assay (ELISA) under conditions described elsewhere {Calzolari, et al. 1995}. Goat anti-cat IgG conjugated to horse radish Peroxidase (Jackson Immunoresearch Laboratories, No. 102-035-003, Lot 63421) was used as conjugate. Absorbance of the samples was determined in a Dynatech MR 7000 micro ELISA reader.

3.5.4. Cytokine analysis to determine the effect of IFN Ω:

2 X 100 µl blood + 300 µl Lysis buffer for mRNA extraction were collected and sorted in -80°C immediately. Before the extraction of mRNA was initited, the stability of the mRNA over 10 freezing and thawing cycles was evaluated. To this end, RNA were extracted from 1 cat and divided into 3 aliquots, the 3 samples were treated as follows:-

Sample 1 was frozen at -80°C.

Sample 2 was subjected to 10 cycles of freezing at -20°C and thawing.

Sample 3 was stored at +4°C.

After this pretreatment mRNA was extracted from each sample according to manufacturer's protocol (MagNA Pure LC mRNA Isolation Kit, Roche GmbH, Mannheim, Germany) All three samples were assayed for GAPDH mRNA by TaqMan {Leutenegger, et al 2001}. The Ct value of the cycled samples was less than 1 Ct value higher (Pkw = 0.7557). Depending on this results, we decided to freeze and thaw the RNA preparations for assaying cytokine mRNA and the samples collected on one day were tested in parallel. Later, mRNA was extracted from 10^6 WBCs from all collected samples as described above, labelled and stored immediately at -80 °C.

To determination the cytokine mRNA activity in the cat samples, the samples which were collected on one day were pipetted onto one plate in parallel together with a minimum of five calibrators and tow negative controls. The mRNA results of the samples are expressed in relation to the calibrators.

3.5.5. Clinical chemistry:

Heparinized blood samples were collected and after centrifugation (1000x g, 10 min.) plasma was analyzed for ALAT. ASAT, AP, lipase, bilirubin, glucose, urea, creatinin, protein, albumin, chlolestrin, sodium, potasium, chloride, calcium and phosphate parameters in a Cobas integra 8000 instrument using standardized test procedure (Roche diagnostics, Rotkreuz, Switzerland).

3.5.6. Virus isolation:

PBMCs were isolated from EDTA venous blood on a weekly basis. Cells were grown in culture under conditions identical to those described previously {Lutz, et al. 1988}. To demonstrate virus present in the supernatant, TNA were extracted as mentioned above and presence of FIV specific RNA was demonstrated by real- time RT-PCR under conditions mentioned in section 3.5.2.

3.6. Viral load detection from Saliva:

To determined presence of FIV RNA and its concentration, salivary swabs were collected weekly. RNA was extracted and real- time RT-PCR was demonstrated as described above.

3.7. Primers and probes:

3.7.1. TagMan primers and probes:

Primers and probes used in this study have been described elsewhere. They are compiled in table 2.

Table (2) primer- and probe sequences used in this study:

Primer	Sequence (5` → 3`)
FIV 552f	GCCTTCTGCAAATTTAACACCT
FIV 672r	GATCATATTCTGCTGTCAATTGCTTT
fGAPDH.57f	GCCGTGGAATTTGCCGT
fGAPDH.138r	GCCATCAATGACCCCTTCAT
IL10.182f	TGCACAGCATATTGTTGACCAG
IL10.257r	ATCTCGGACAAGGCTTGGC
IL12.253f	TGGCTTCAGTTGCAGGTTCTT
IL12.333r	TGGACGCTATTCACAAGCTCA
Probe	**Probe sequence (5` → 3`)**
FIV582 p	TGCGGCCATTATTAATGTGGCCATG
fGAPDH.77p	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA
IL10.209 p	ACCCAGGTAACCCTTAACCTCCAGCA
IL12.283 p	CGGTTTGATGATGTCCCTGATGAAGAAGCT

For all the sequences studied, the sense and antisense primers were placed in two consecutive exons of the gene. The probe spanned the junction of two exons, covered by the forward and reverse primer. To avoid the requirement of a separate extension step, short PCR products were selected; the length of the amplified sequence was below 100bp. Primers and probe sequences are listed in Table 2.

For feline IL-10, IL-12p40 and GAPDH, the detection system which was previously described was applied {Leutenegger, et al. 1999, 2001}.

3.7.2. Primers and probes optimisation:

To determine the optimal primer and probe concentrations that yield the maximum ΔR_n and minimum Ct, the recommendations published for the ABI prism 7700 instrument (ABI, Baar, Switzerland) were followed.

3.7.2.1. Optimal primers concentration:

Three different concentrations 50, 300, and 900 nM were used to determine the best primer concentrations amplifying the RNA standards of GAPDH and the Cytokines IL10 and IL12. Optimal concentration for GAPDH forward and reverse primers was found 300 nM. For IL-10 optimal forward primer was found to be 300 nM and for reverse primer was found 900 nM.

For IL-12p40 optimal forward and reverse primers were found to be 900 nM and 600 nM respectively.

3.7.2.2. Optimal probes concentration:

Three different concentrations 100, 200, and 300 nM were used. Optimal concentrations were found to be 250 nM for GAPDH, IL-10 and IL-12p40.

3.8. Cytokine cloning and sequencing:

A plasmid standard for FIV RNA PCR was prepared as described earlier {Leutenegger et al., 1998}. Briefly, feline plasmid (pMOK; Mologen AG) transformed in E.coli (JM-109 competent cells from Novagene, Darmstadt, Germany), Topo vector were cut, purified, ligated and transformated.

Primers were designated. PCR conditions were as follows: Denaturation 96°C for 30s; annealing 96°C for 10s; primer extension 50°C for 5s; 60°C for 4min;25 cycles. Fragments were cloned by a TA cloning strategy into a TOP 10 F`vector (Invitrogen, Madison, WI, USA) propagated in E coli and sequenced with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Baar, Switzerland) Dilutions of the sequenced, linearized, in vitro transcripted, and purified plasmid were used as standards to test for the analytical sensitivity in the range from 10^1 to 10^9 copies. Aliquots of dilutions were frozen at -80°C and used in real- time RT-PCR only once.

3.9. Quantitation of GAPDH and cytokine expression by real- time RT-PCR:

To quantitate mRNA expression of the cytokines of interest, the procedures published by Leutenegger were used {Leutenegger et al. 2002}.

Cytokine analysis from PBMCs:

PBMC were isolated at week 0 and week 8 p.i, and for each cat 3 cell culture wells with $5x10^5$ cells were set up.

1) Unstimulated
2) ConA stimulated: 10 µg/well
3) LPS stimulated: 1 µg/well

Cells were harvested after 24 h incubation, 300 µl mRNA lysis buffer were added and stored at –80ºC till using mRNA were extracted with MagNA Pure (mRNA Isolation Kit I, mRNA I cells protocol). Real- time RT-PCR analysis of the mRNA and RNA standards was carried out in AB 7700 instrument.

Table 3: Final probe- and primers concentrations used in this study:

Standard	Probe	Copy number	Primer F	Primer R	Slope
FIV-DNA	25 µM	10^{3} - 10^{8}	0.3 µM	0.3 µM	-3.5
FIV-RNA	25 µM	10^{1} - 10^{8}	0.9 µM	0.3 µM	-3.5
fGAPDH	25 µM	10^{4} -10^{8}	0.3 µM	0.3 µM	-3.4
IL-10	25 µM	10^{3} -10^{7}	0.3 µM	0.9 nM	-3.5
IL-12p40	25 µM	10^{3} - 10^{8}	0.9 µM	0.6 µM	-3.6

3.10. Analysis of measured values:

Statistical evaluation of the results of the entire study was performed using a standardised procedure. All results were first tested for presence of normal distribution using the Kolmogorov-Smirnov test and subsequently further analysed depending on the presence or absence of normal distribution. Data showing normal distribution for all time points were tested by ANOVA for repeated measures, followed by a Bonferroni correction with a significance level of 5 %. Paired groups were evaluated for significant differences by the Student's T-test. Data not showing normal distribution in one or more time points were evaluated for paired groups, each time point separately by the Mann Whitney U-test. P-values in the legends of fig.s and tables were indicated by colours: yellow: significance ($p < 0.05$), green: tendency to significance ($0.05 < p < 0.08$); brown: no significance.

4. Results:

4.1. SPF status of the cats at the beginning of the experiments:

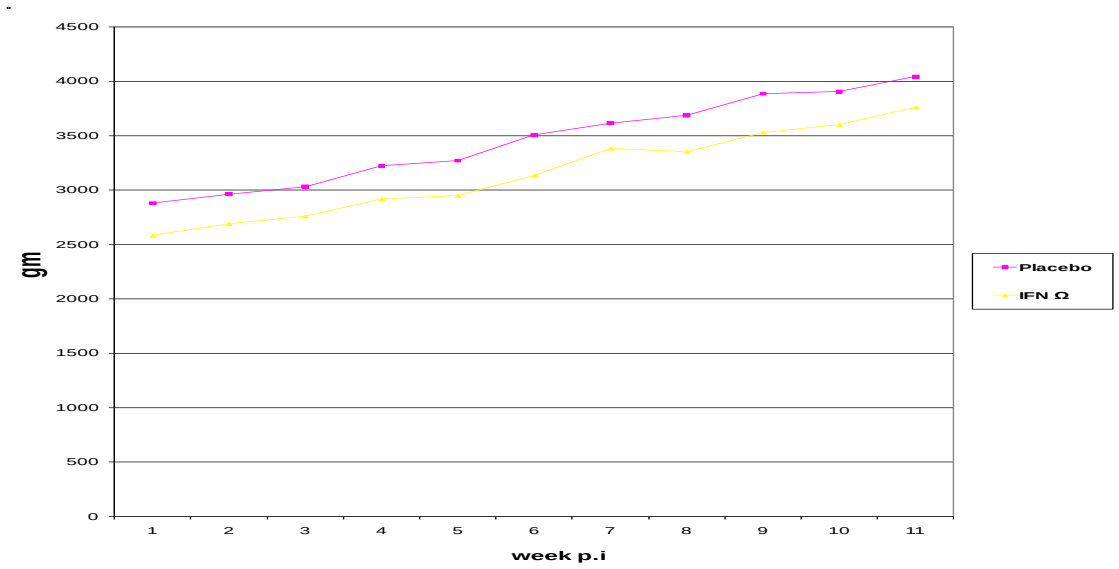
Immediately, after arrival of the animals at our facility, all cats were tested by serology and by PCR and real- time RT-PCR, respectively for absence of infection by FIV, FeLV, FCoV, FCV, FHV, and FPV and all cats were found to be negative.

4.2. Clinical signs:

All cats were examined clinically once per week, and the following parameters were determined: weight gain, temperature, and clinical signs (food intake, lymphnode size, mucousmembrane, heartbeat, respiratory rates, abdominal palpation and defecation).

4.2.1. Weight gain:

The two groups started at slightly different mean weights at the beginning of the experiment. This can be explained by the fact that assignment to groups was done according to the origin of the cats; litter mates were assigned to different groups in order to minimize genetic relatedness. As a consequence, the groups were not formed according to weight which explains the weight difference. Weights developed more or less parallel, independent of interferon treatment and challenge infection (fig. 3). Interestingly, at several time points the differences in weight were significant as indicated at the bottom of fig. 3.



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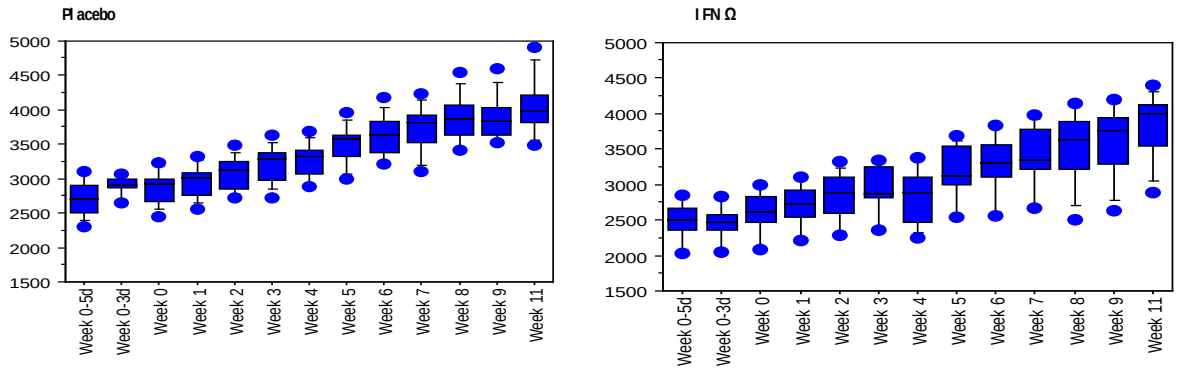


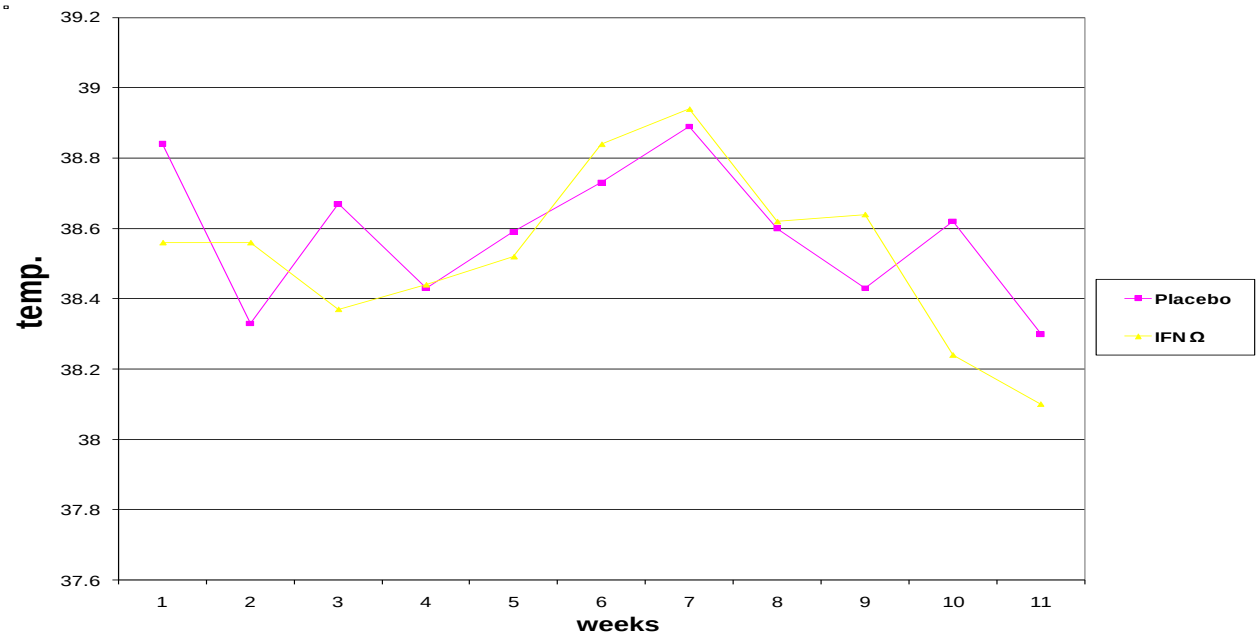
Between groups (p-values):

Weeks	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	W11
Placebo IFN Ω	0.0595	0.0001	0.0249	0.0367	0.0618	0.0415	0.0088	0.0256	0.0598	0.0577			

Fig. 3: Development of body weight during the experiment

4.2.2. Body temperature:

In both groups the mean starting body temperature was 39.2°C. After an initial drop, the temperature varied around a median of about 38.5°C. There was not a single week with significant difference in temperature and no time association with treatment and challenge. One animal showed increased body temperature (41.2°C) in week 6; the cause of the fever was not determined. The results are displayed in fig. 4.



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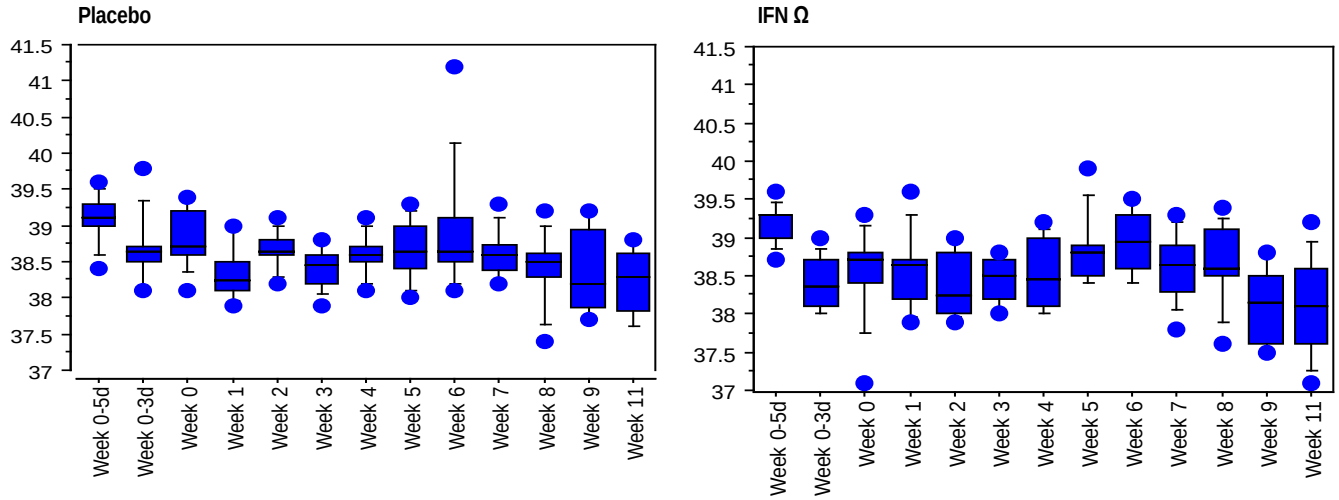


Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω					0.6300								

Fig. 4: Body temperature measurements during the experiment

4.2.3. Clinical well-being:

All cats were clinically evaluated for abnormal behavior and increased lymph nodes once a week. Interestingly, all cats appeared to be clinically healthy independently of the treatment and the challenge infection. One animal (the same as the one showing increased body temperature) showed depression from week 6 to 11. In week 11 a second animal showed depression.

4.2.3.1. Anorexia:

Weeks	W 0	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 11
No. of cats	0/20	0/20	0/20	0/20	0/20	0/20	1/20	1/20	1/20	1/20	2/20

4.2.3.2. Lymphnode increase of size:

L N	W 0	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 11
Mand.	0/20	0/20	2/20	5/20	8/20	12/20	15/20	17/20	20/20	20/20	20/20
Pop.	0/20	1/20	1/20	3/20	6/20	9/20	11/20	15/20	15/20	18/20	18/20

4.2.3.3. Redness of the eyes:

W0	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 11
0/20	0/20	0/20	0/20	2/20	2/20	3/20	3/20	5/20	6/20	6/20

4.2.3.4. Defecation (diarrhea):

W0	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 11
0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	1/20	1/20	1/20

4.2.3.5. Ocular discharge:

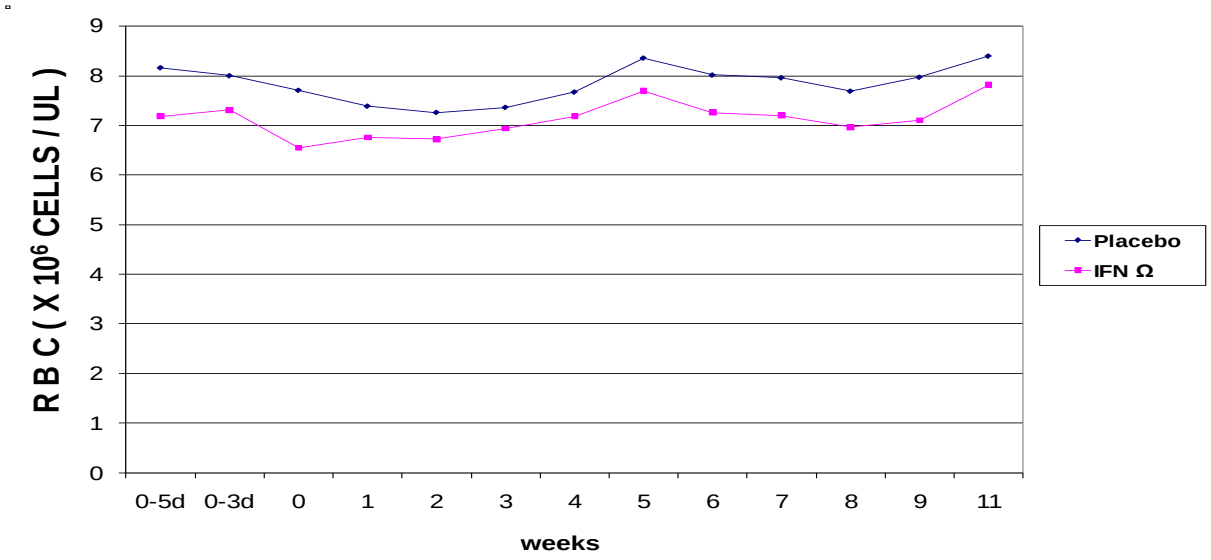
W1	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 11
0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	1/20	1/20	2/20

4.3. Analysis of blood samples:

4.3.1. Hematology:

4.3.1.1. Erythrocyte counts:

Between the two groups the erythrocyte counts differed significantly on several occasions (fig. 5). Animals of the IFN Ω group generally showed lower red cell counts than animals of the placebo group. As the difference was already present on the first day of blood collection, it was concluded that this was due to random parameters as assignment to the two groups of the cats were done on the basis that animals of the same litter were randomly distributed to the two groups in order to minimize genetic relationship within each group.



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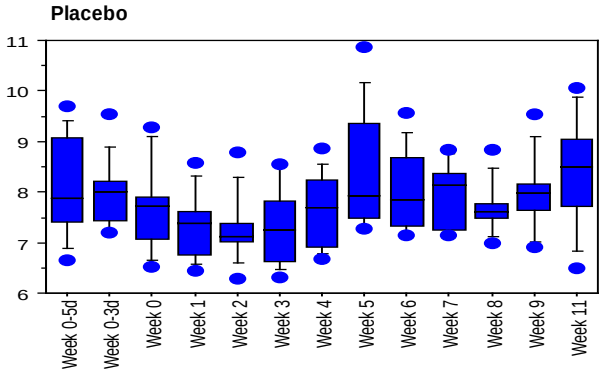
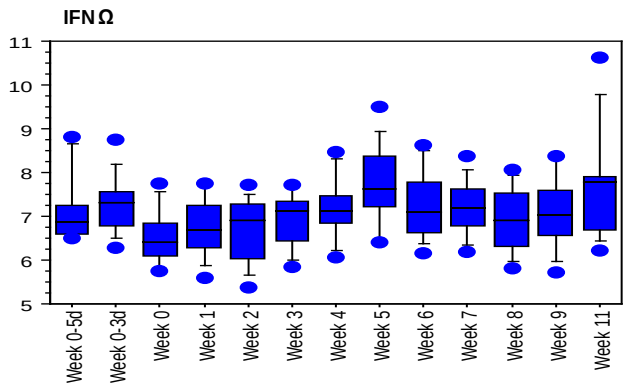




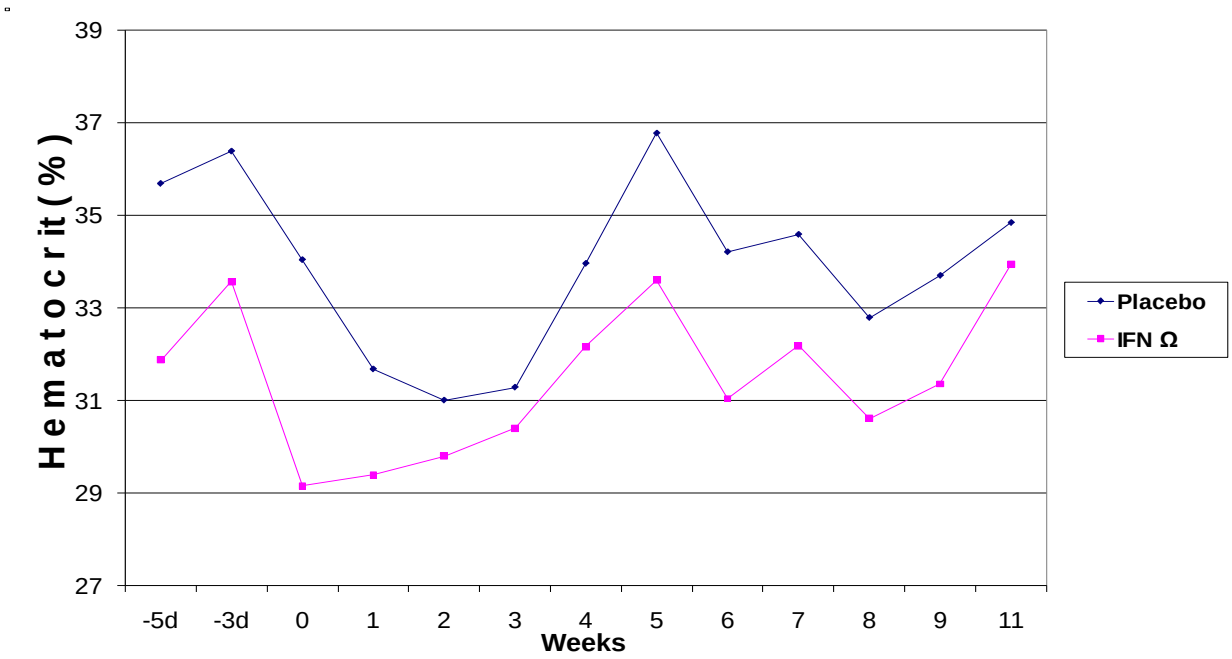
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω	0.0436	0.032	0.0034	0.0593					0.0482	0.0181	0.0263	0.0309	

Fig. 5: Erythrocyte counts during the experiment

4.3.1.2. Hematocrit:

As already observed for the erythrocyte counts, haematocrit values of IFN Ω group were consistently lower than those of placebo group (fig. 6). However, for the haematocrit only two time points displayed significant differences (week 0 and week 6). Again, the difference was already observed at the beginning of the experiment and therefore attributed to the assignment of the cats to the different groups and not to aspects of treatment and challenge.



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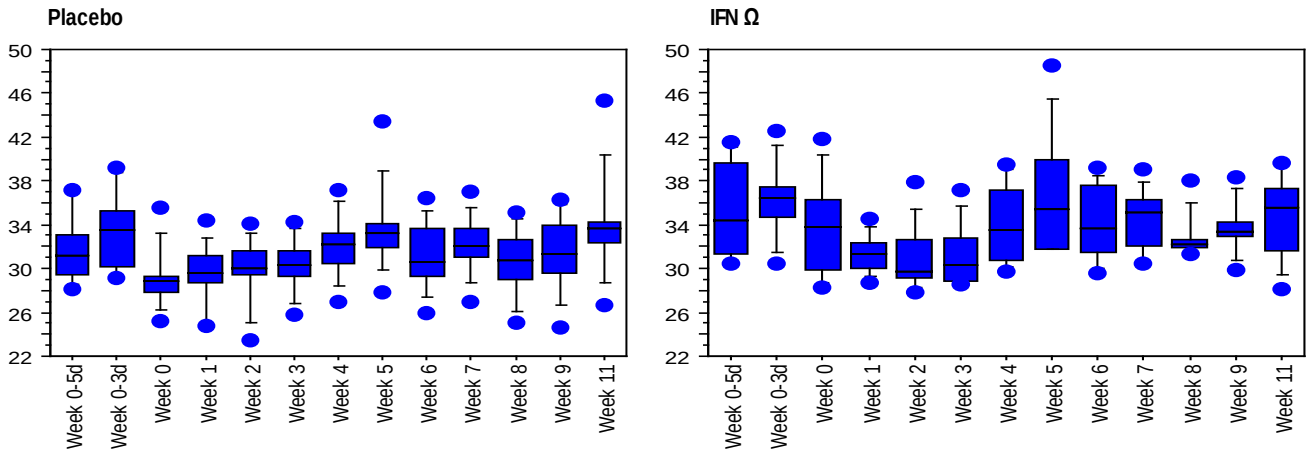


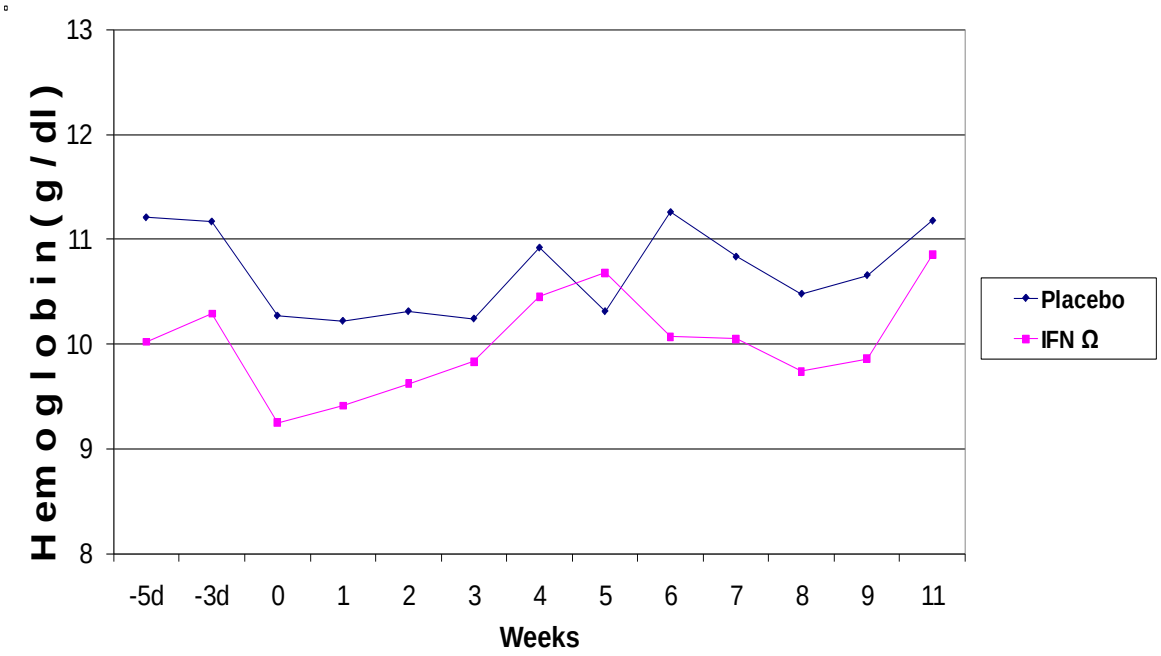
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω	0.0653		0.0069	0.0723					0.0373	0.0675			

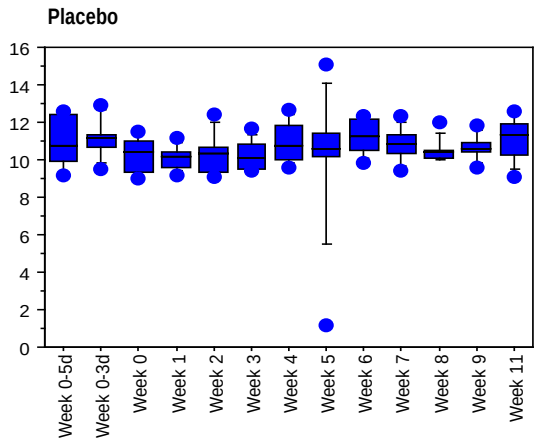
Fig. 6: Hematocrit counts during the experiment

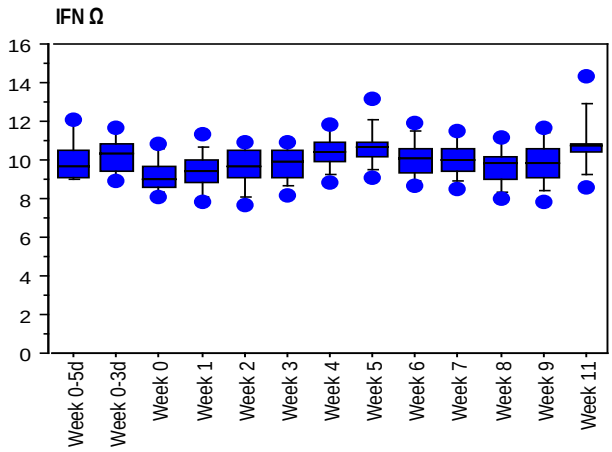
4.3.1.3. Hemoglobin:

Again, and as seen for red cells and haematocrit, animals of the IFN Ω group had lower hemoglobin values than animals in the placebo group (fig. 7). The differences were significant on the identical time points (week 0 and week 6) as seen before for the haematocrit. In week 5, two animals of the placebo group exhibited extreme variation in the values, which are most likely due to technical errors that occurred during the analysis.



Frequency distribution:





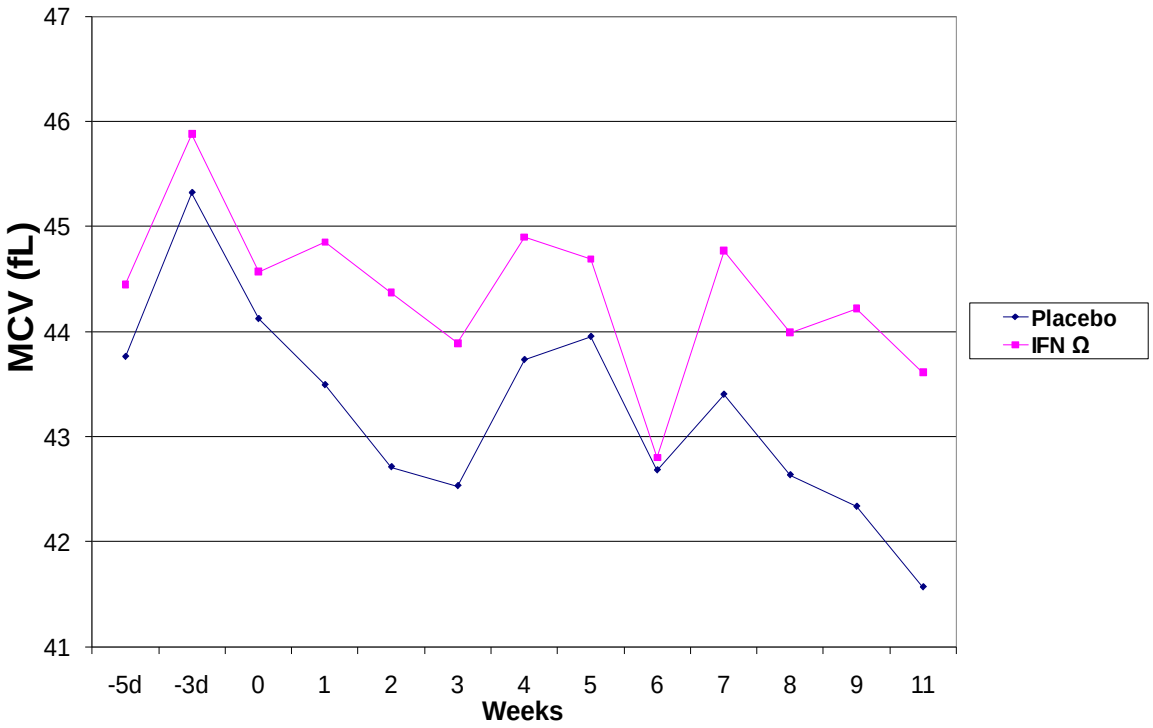
Significance between groups (p-values):

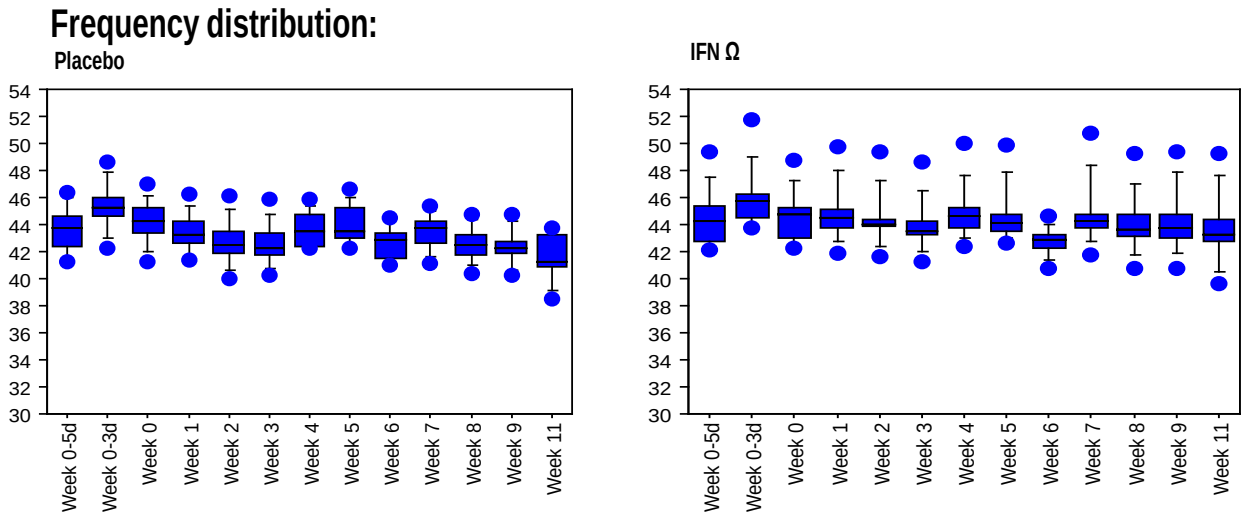
weeks	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0563	0.0185	0.0680					0.0109	0.0670	0.0671	0.0744	

Fig. 7: Hemoglobin values measurements during the experiment

4.3.1.4. Mean corpuscular volume (MCV):

As seen for the other red cell parameters, the mean MCV values showed systematic, although not significant differences (fig. 8).





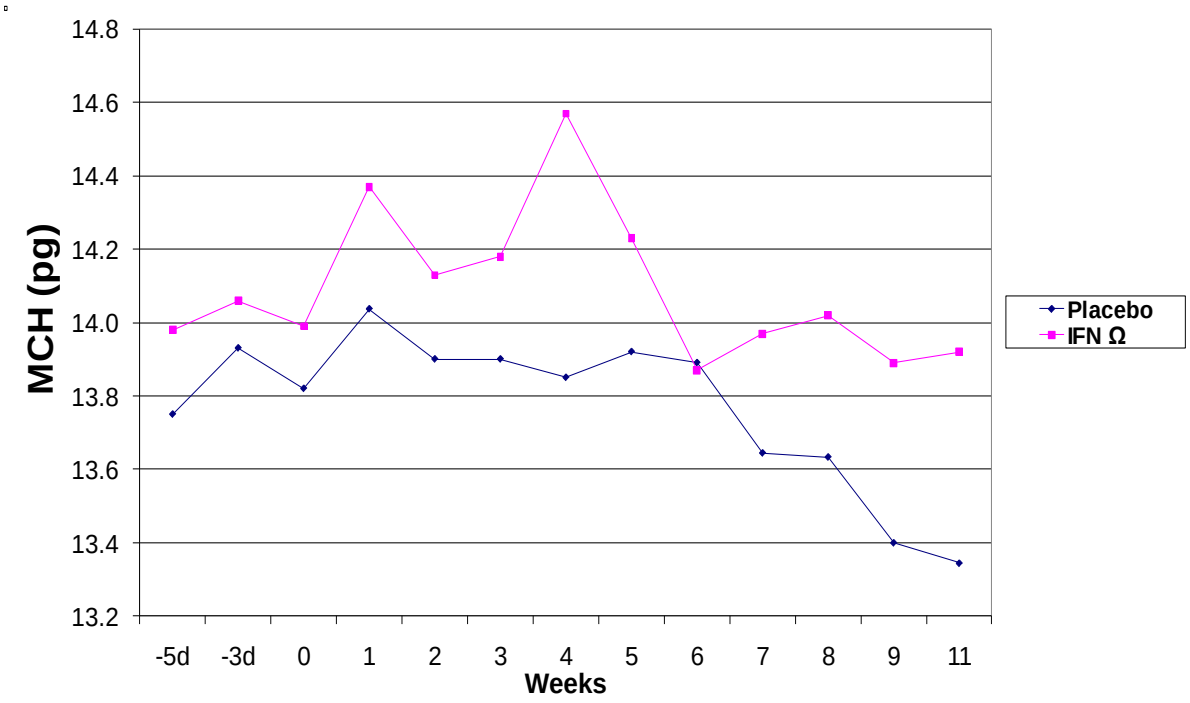
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω				0.0753	0.0594							0.0452	0.0605

Figure 8: MCV parameters during the experiment

4.3.1.5. Mean corpuscular hemoglobin (MCH):

In fig. 9, the course of MCH values is shown. As seen for the MCV results, the MCH results of IFN Ω group were higher than those of placebo group throughout the entire observation period. Differences between the two groups were not significant at any date.



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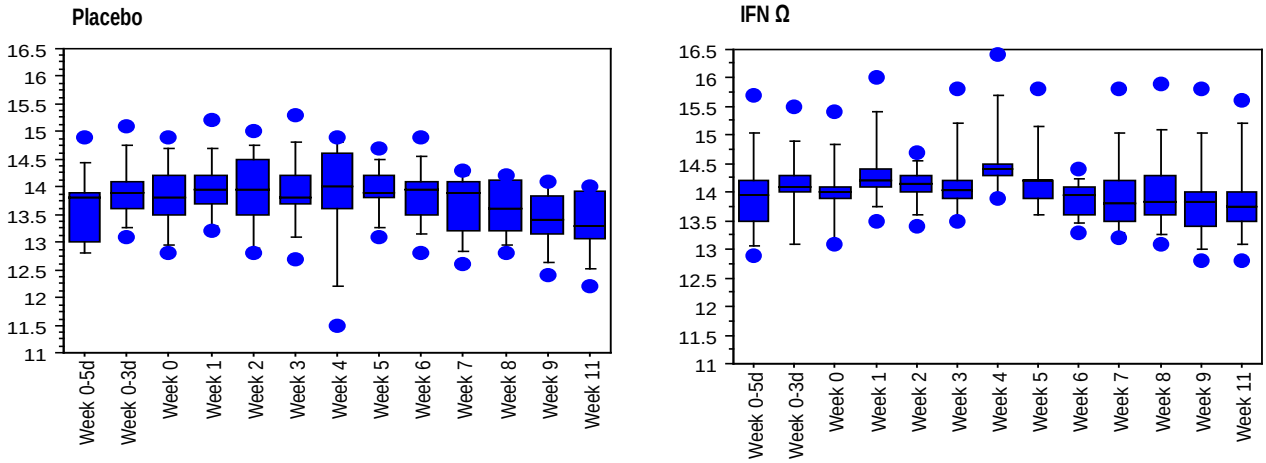


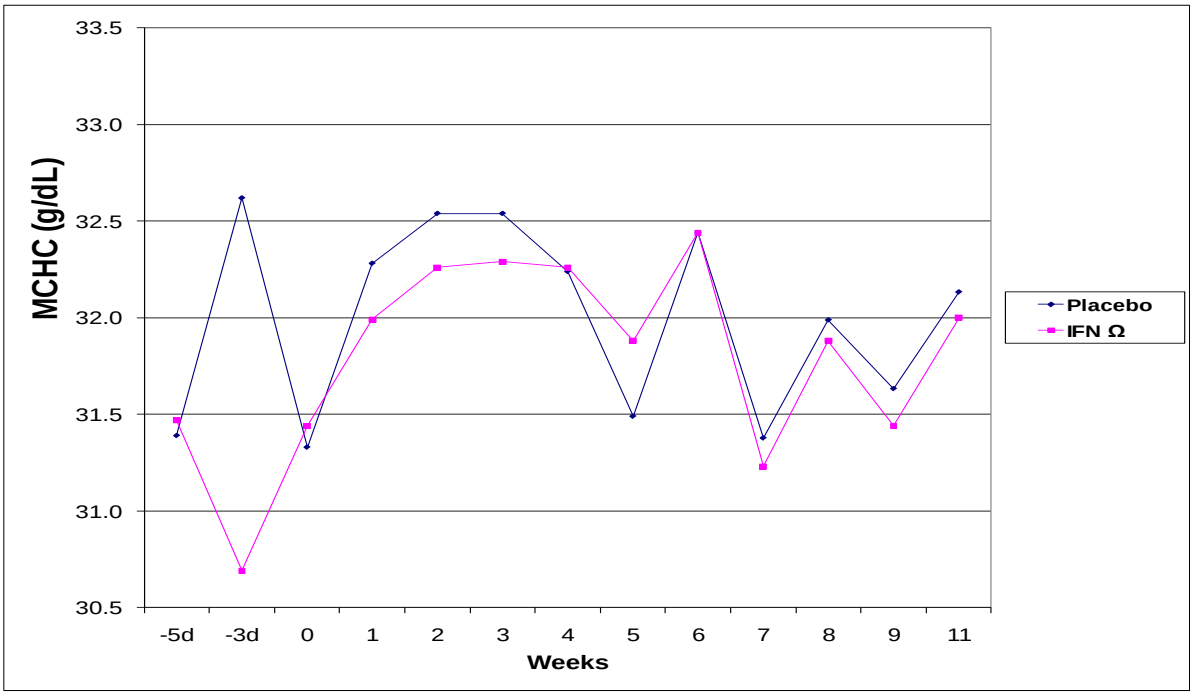
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

Fig. 9: MCH values during the experiment

4.3.1.6. Mean corpuscular hemoglobin concentration (MCHC):

The MCHC values did not show any significant difference throughout the course of the entire experiment (fig. 10). MCHC is tightly controlled displaying only minimal variation. The one animal in the placebo group displaying an MCHC of 45.8 g / dl most likely is the consequence of a false measurement.



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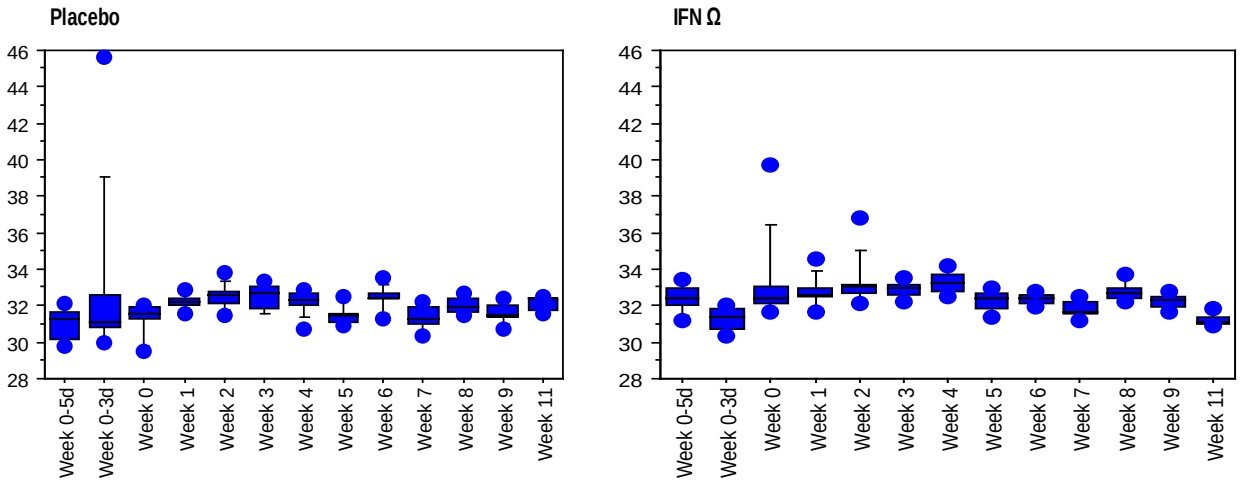


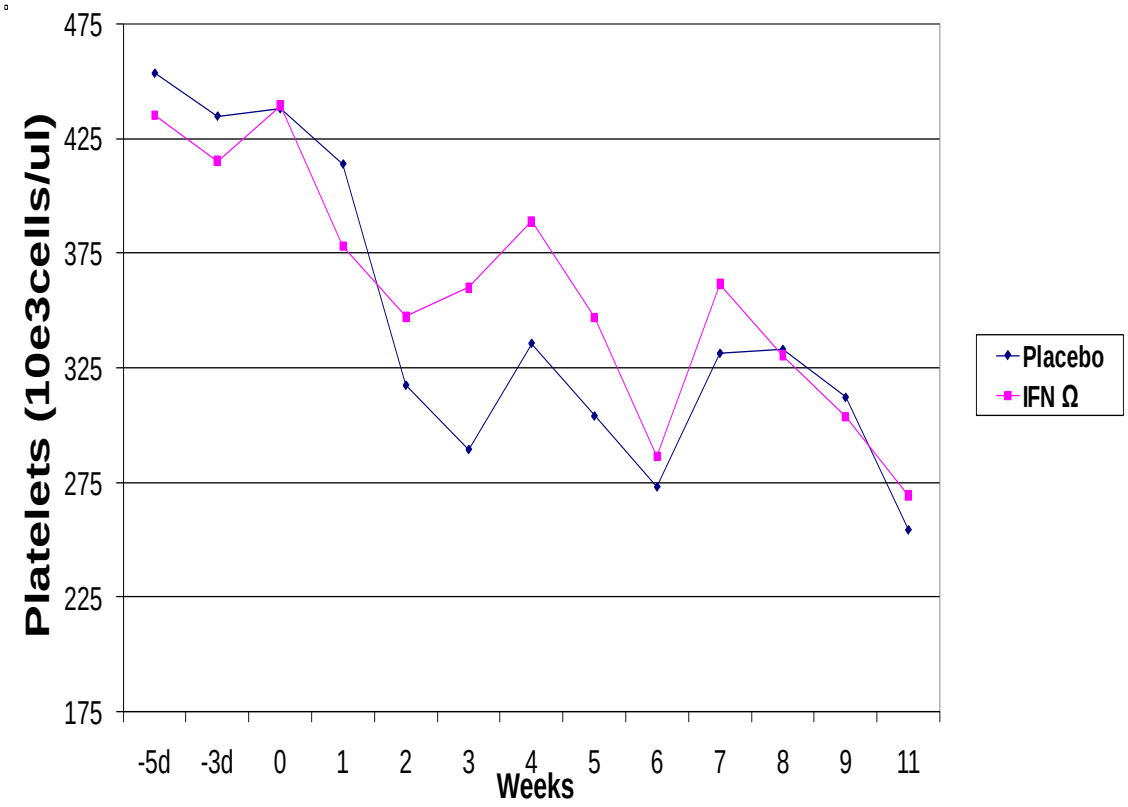
Significance between groups (p-values

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

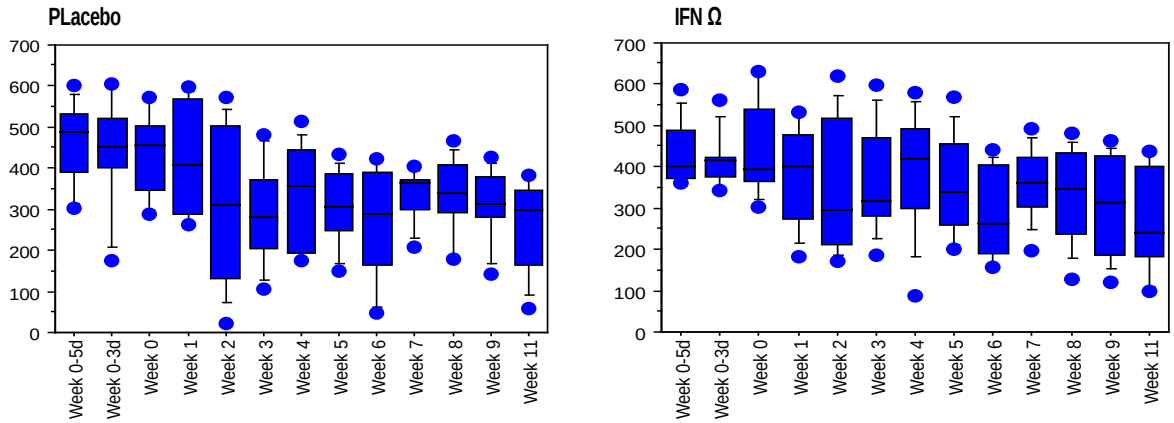
Fig. 10: MCHC values during the experiment

4.3.1.7. Platelets:

The platelet counts in both groups steadily declined over the entire observation period. There was not a single date with significant differences of the mean values (fig. 11).



Frequency distribution:



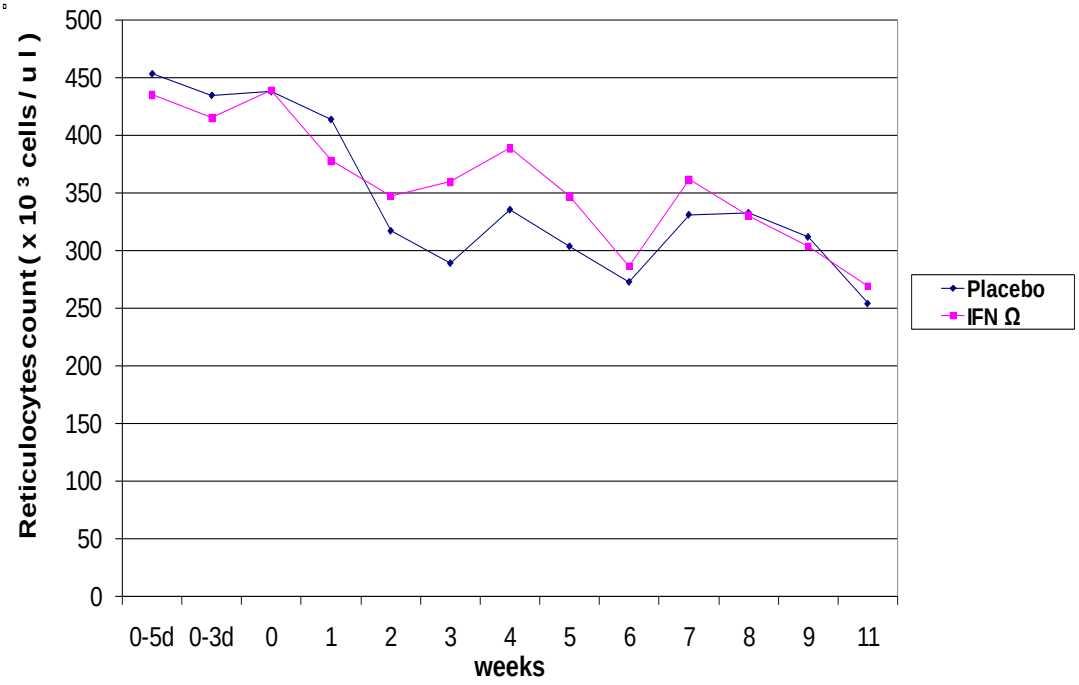
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	W7	w8	w9	w11
Placebo / FN Ω													

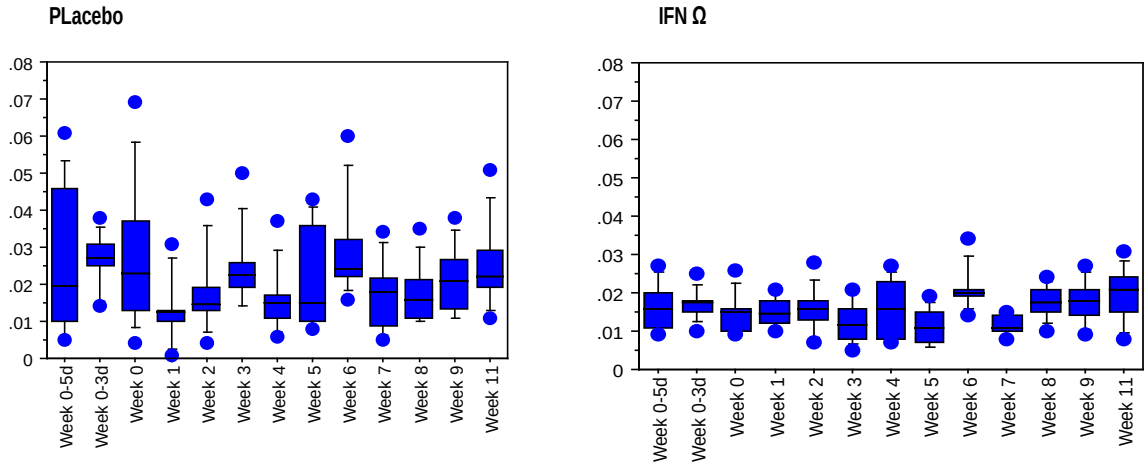
Fig. 11: Platelets counts during the experiment

4.3.1.8. Reticulocytes:

The reticulocytes were measured because it was anticipated that FIV infection might have an effect on the rate of red cell regeneration. Interestingly, the reticulocytes demonstrated a slight but steady decline over the entire observation period (fig. 12) with significant differences on three occasions.



Frequency distribution:



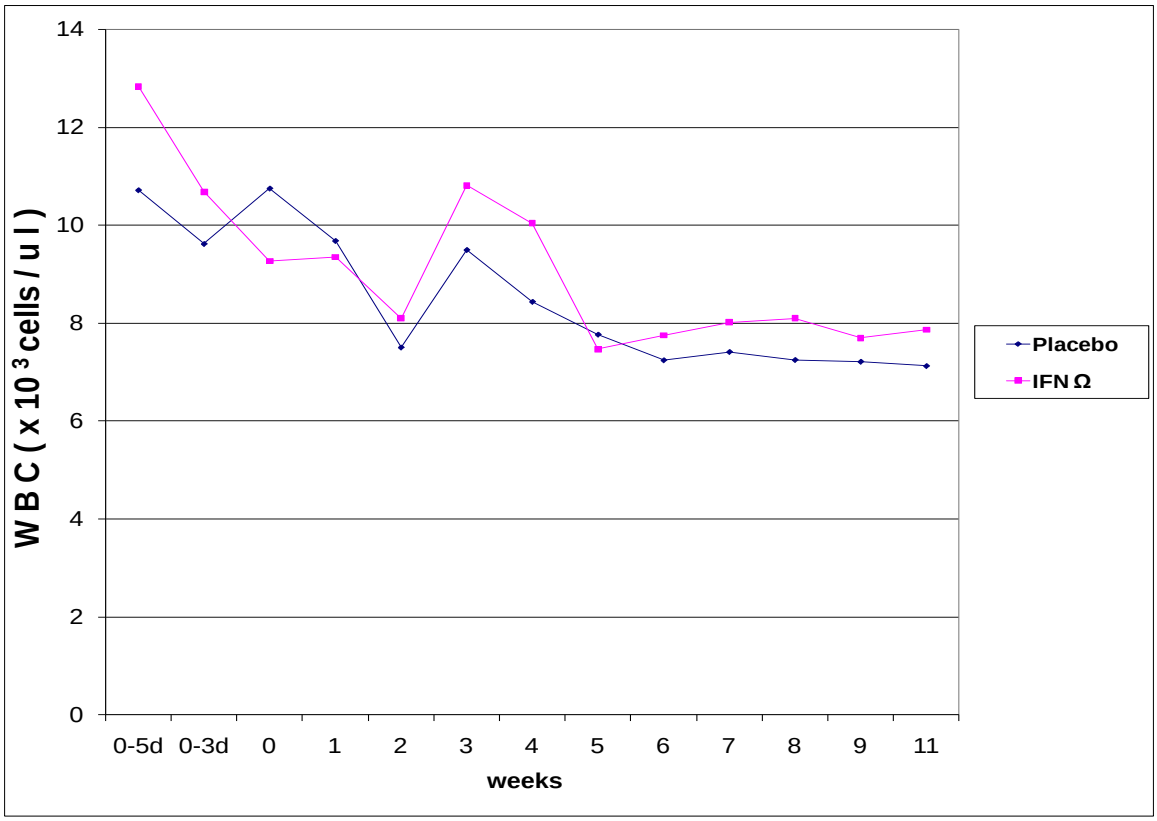
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0016	0.0562			0.0039		0.0358					

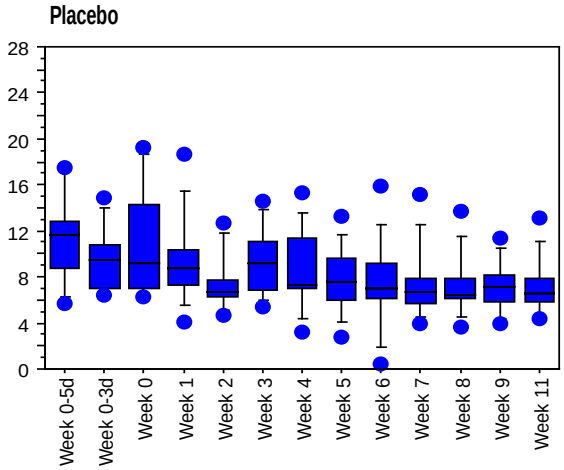
Fig. 12: Reticulocytes measurement during the experiment

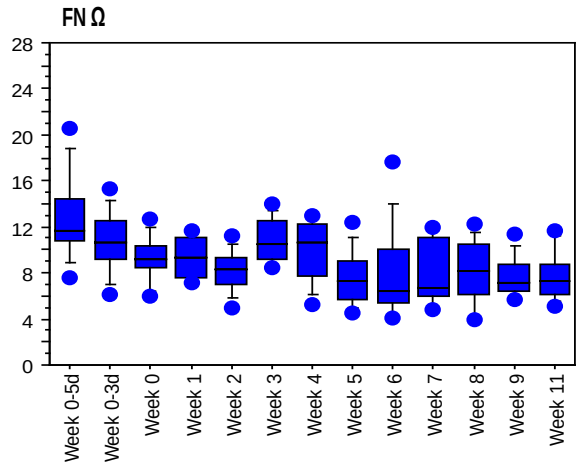
4.3.1.9. Leucocyte counts:

The leucocyte values declined slowly but steadily over the entire observation period; there was not one date with significant differences in the leucocyte concentration (fig. 13).



Frequency distribution:





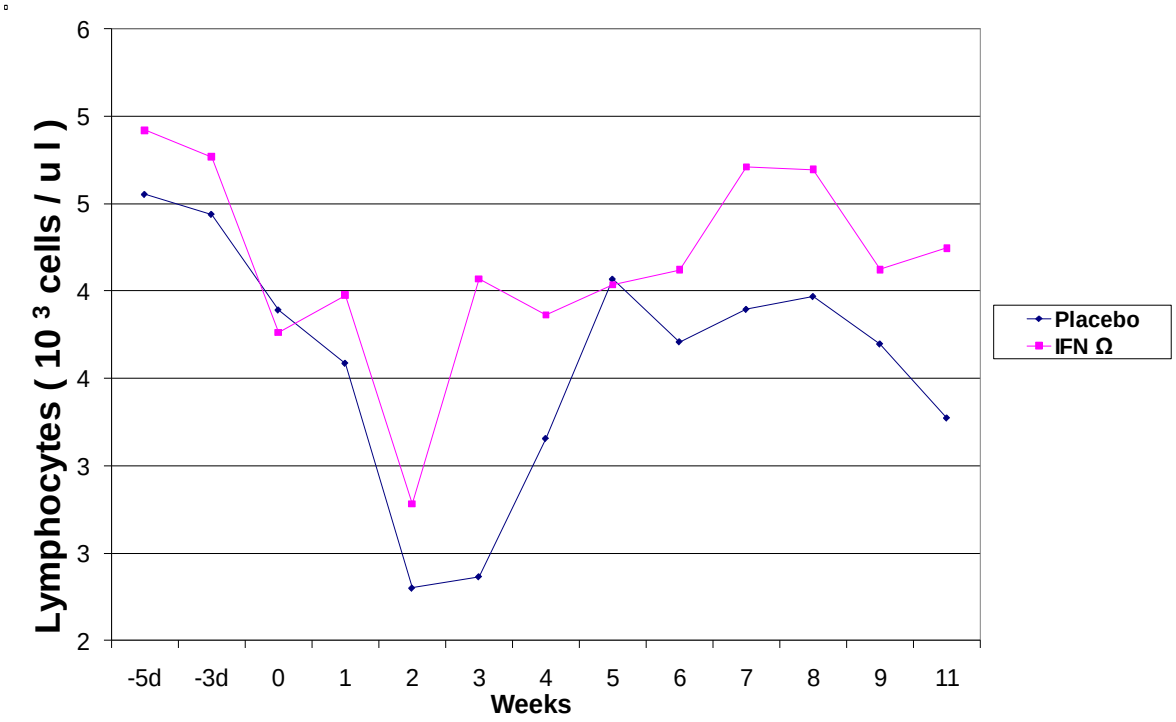
Significance between groups (p-values):

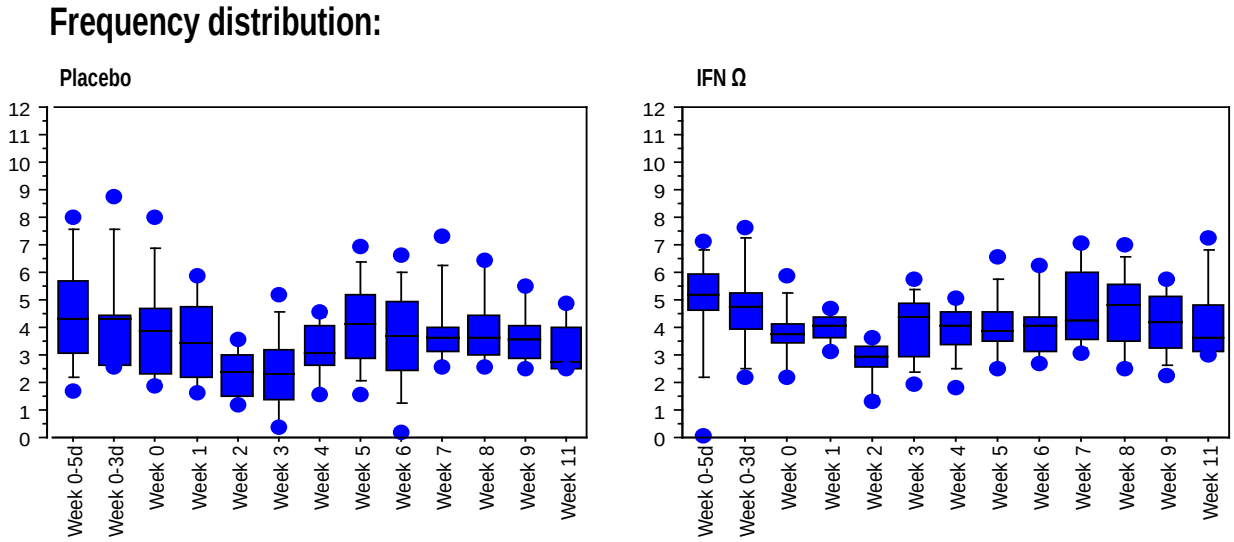
WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

Fig. 13: Leucocyte counts during the experiment

4.3.1.10. Neutrophil counts:

As seen before for the total leucocytes, neutrophil counts did not show any difference between the two groups (fig. 14).





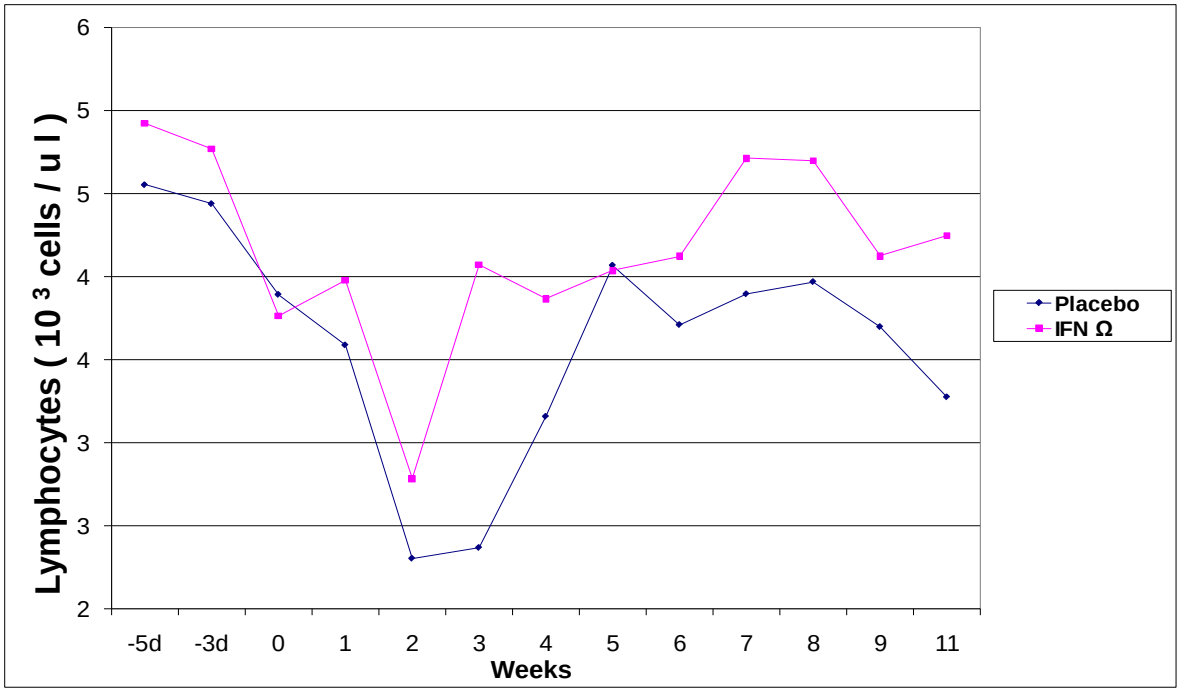
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

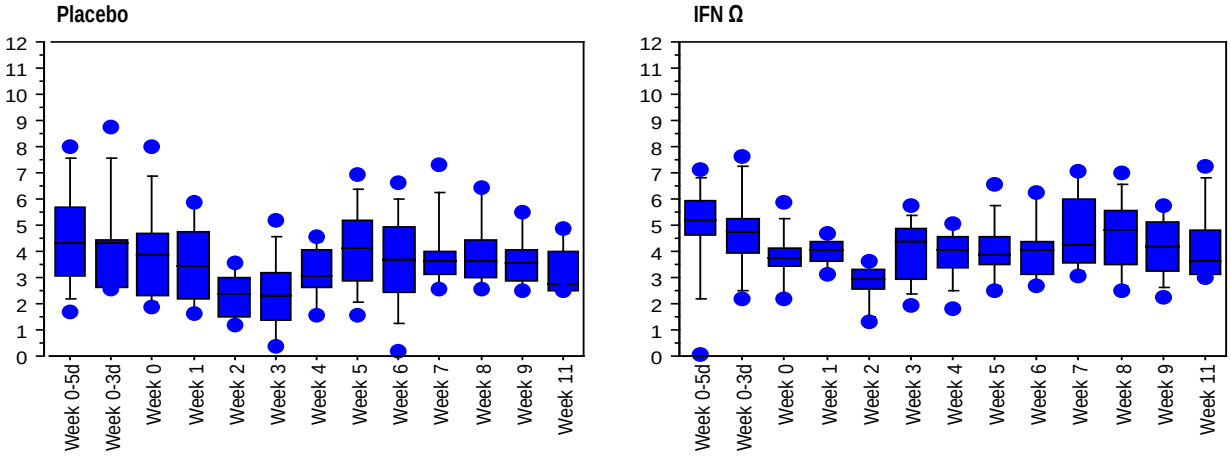
Fig. 14: Neutrophil counts during the experiment

4.3.1.11. Lymphocyte counts:

The mean lymphocyte counts of both groups looked very similar, showing a significant difference only in week 3 (fig. 15). The relatively sharp decline of mean lymphocyte counts in weeks 2 for placebo group cats in week 2 and 3 for IFN Ω group cats reflects FIV infection which usually affects peripheral lymphocyte counts.



Frequency distribution:



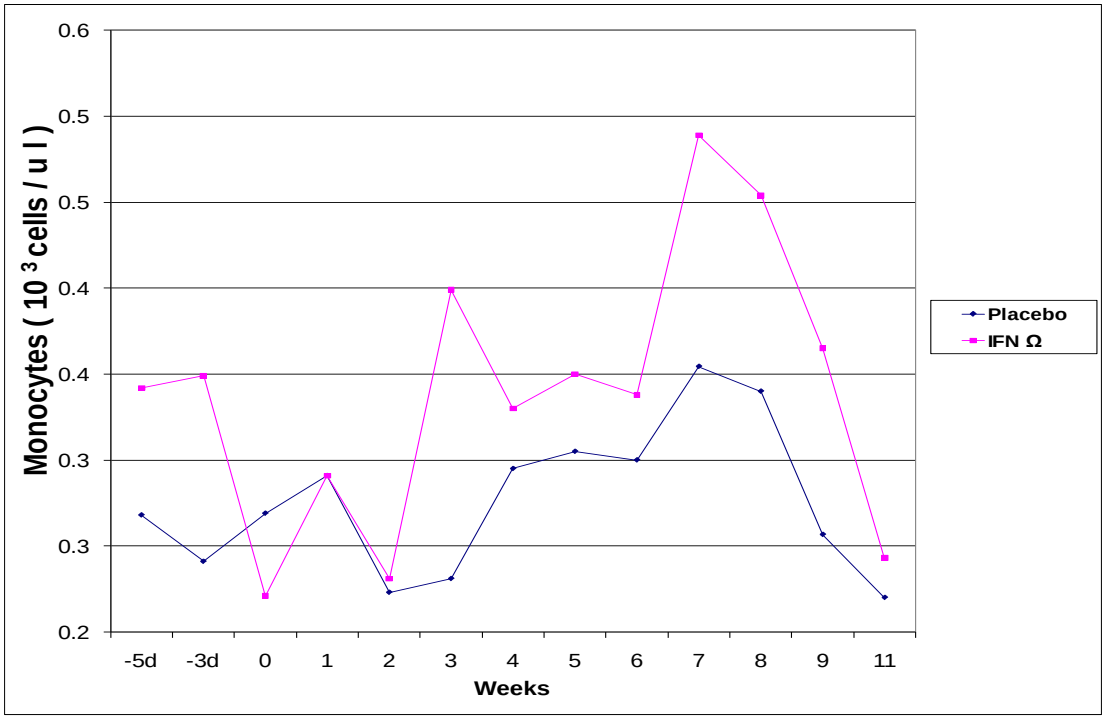
Significance between groups (p-values):

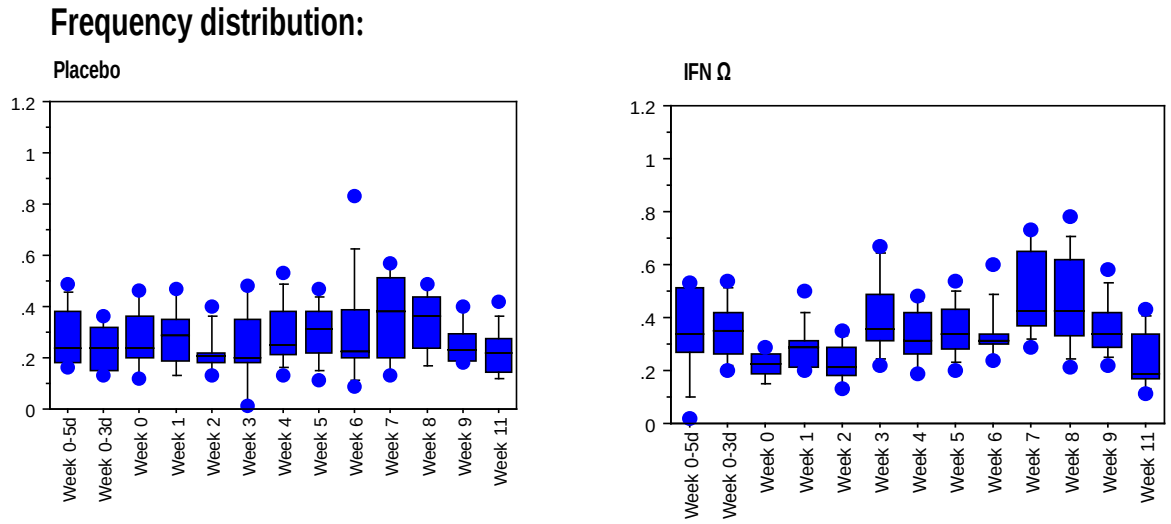
WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω						0.0107							

Fig. 15: Lymphocyte counts during the experiment

4.3.1.12. Monocyte counts:

The monocyte counts of both groups showed some variation but never went beyond the normal reference range (fig. 16). Significant differences in the mean values between the two groups were seen on three occasions (week 0 –3d, week 3, week 9).





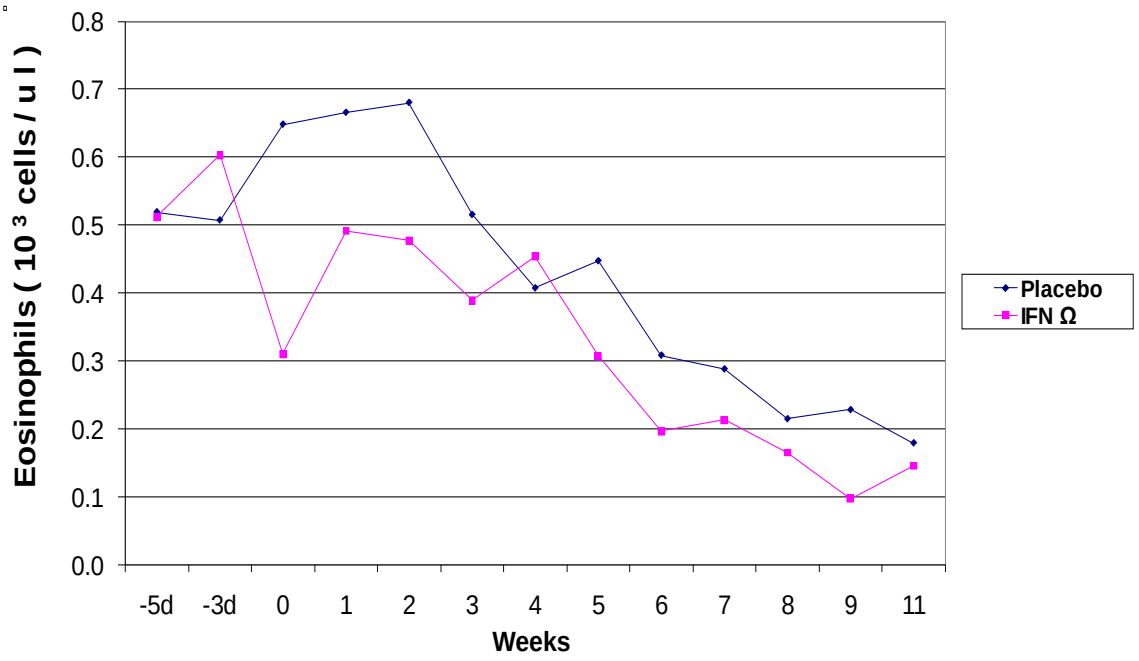
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	W0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0248				0.0250						0.0273	

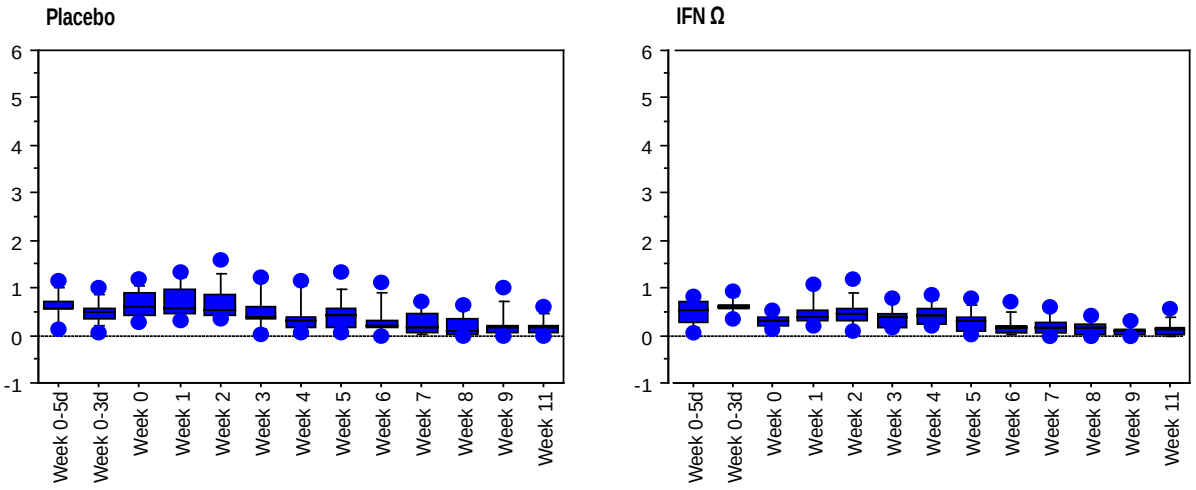
Fig. 16: Monocyte counts during the experiment

4.3.1.13. Eosinophil counts:

As seen before for the total leucocytes and neutrophils, the eosinophil counts showed a steady decrease with time (fig. 17). No significant differences between the two groups were observed throughout the observation period.



Frequency distribution:



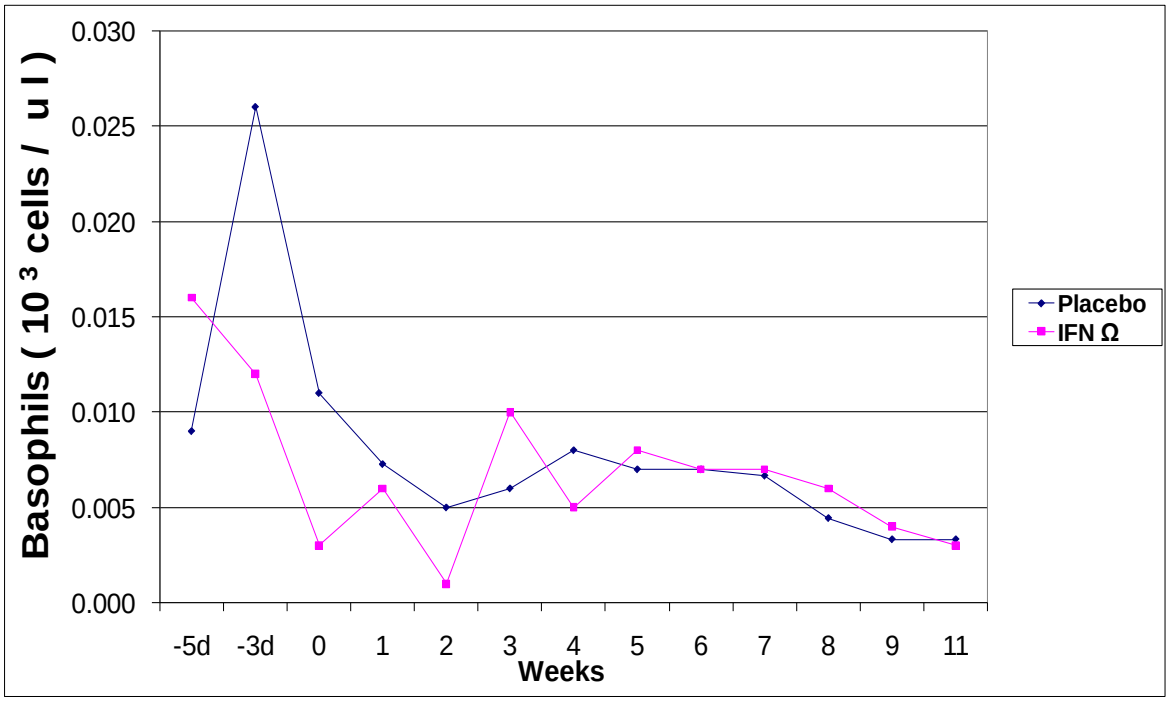
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω			0.0035										

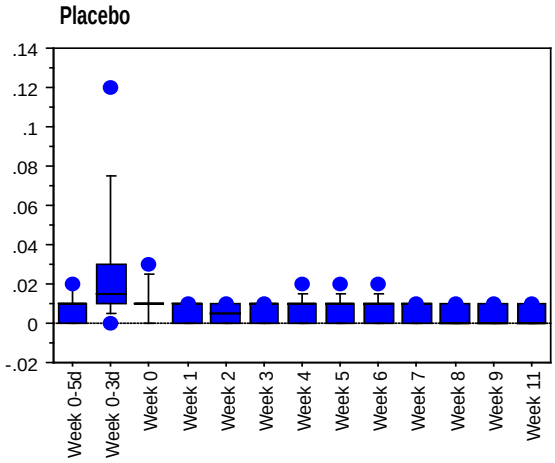
Fig. 17: Eosinophil counts during the experiment

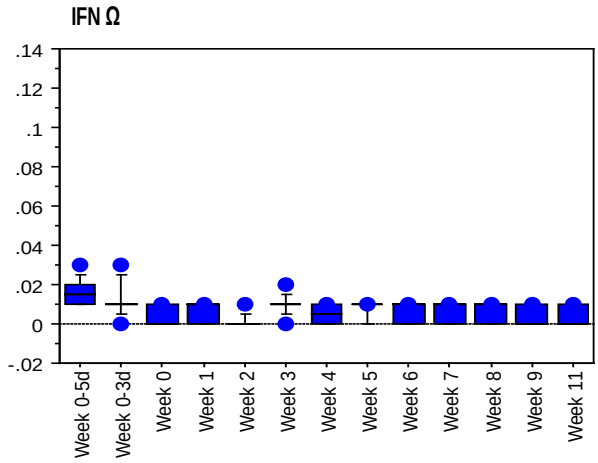
4.3.1.14. Basophil counts:

Basophils were very low in absolute counts and showed only minimal variation (fig. 18). The mean values were statistically different on one occasion (week 0).



Frequency distribution:





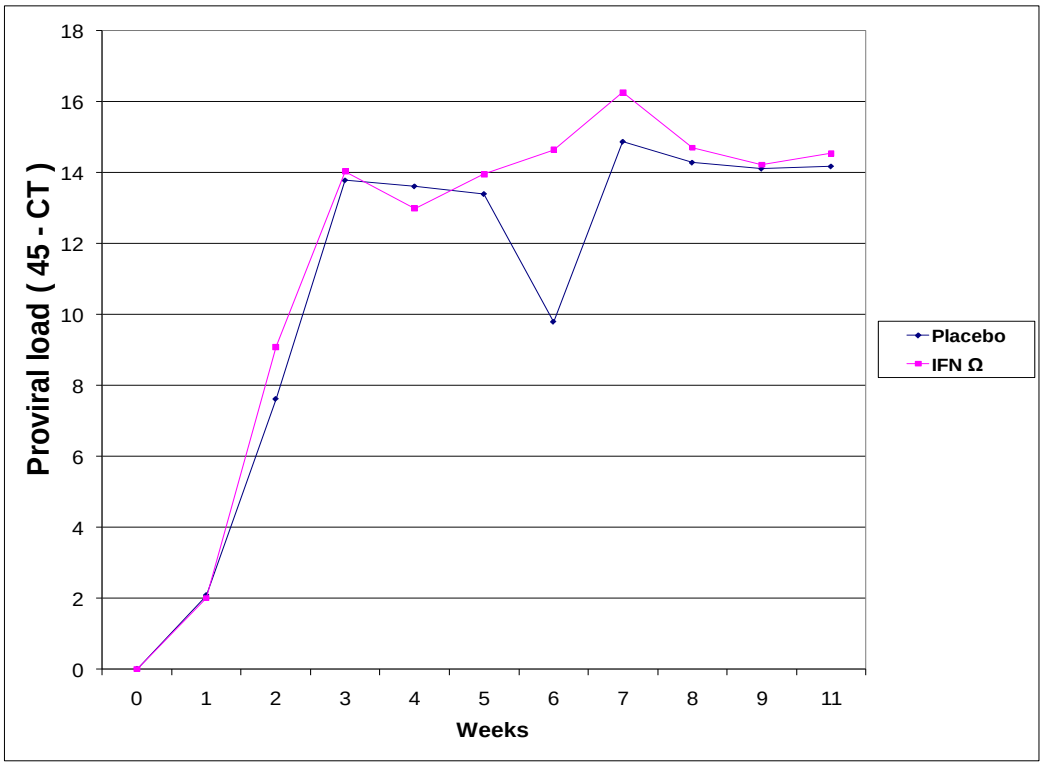
Significance between groups (p-values):

Weeks	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω	0.0757		0.0210		0.0544								

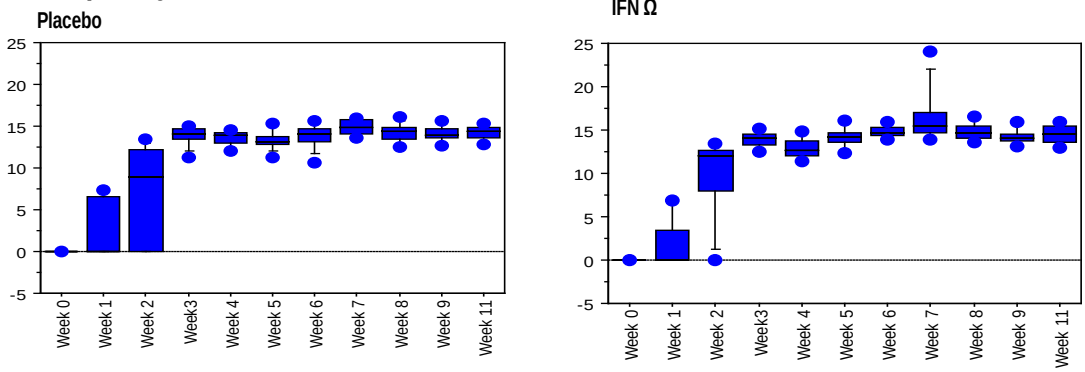
Fig. 18: Basophil counts during the experiment

4.3.2. Proviral load in whole blood:

The proviral load was measured by real- time PCR; the results are given as 45 – the measured CT value (fig. 19). From comparison of the mean values of the two groups it becomes evident that the proviral load is almost identical in both groups. The mean values of both groups did not show any significant difference throughout the observation period.



Frequency distribution:



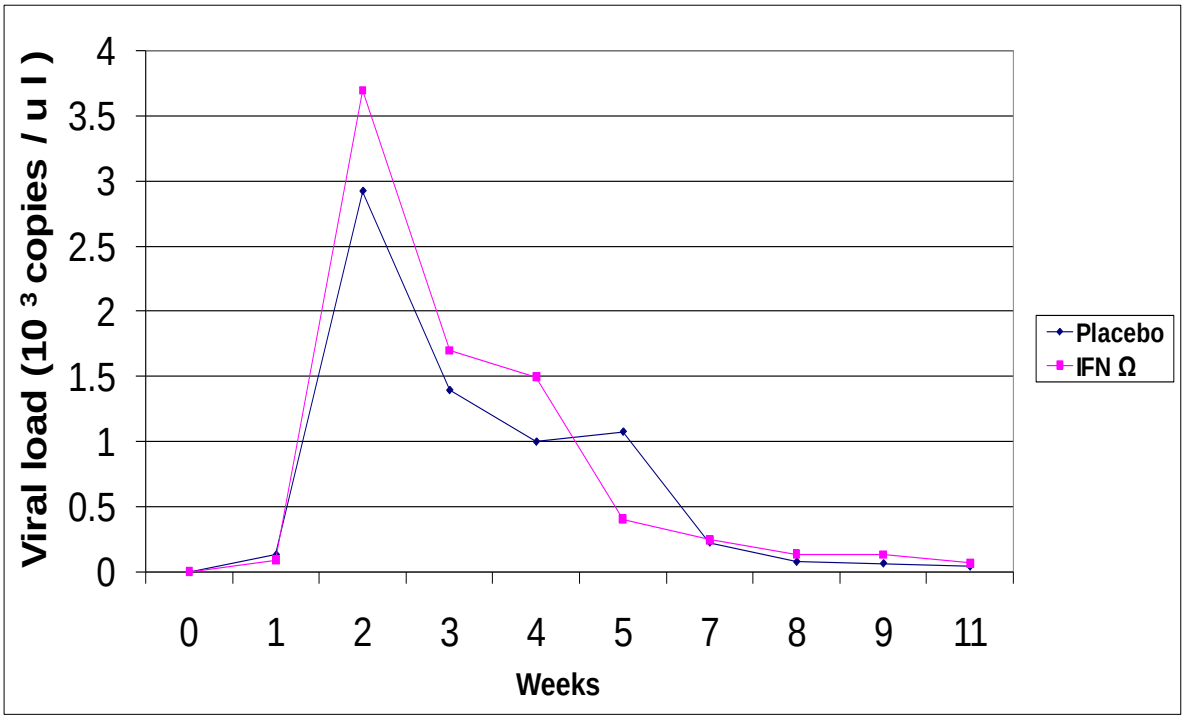
Significance between groups (p-values):

Weeks	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω									0.0560				

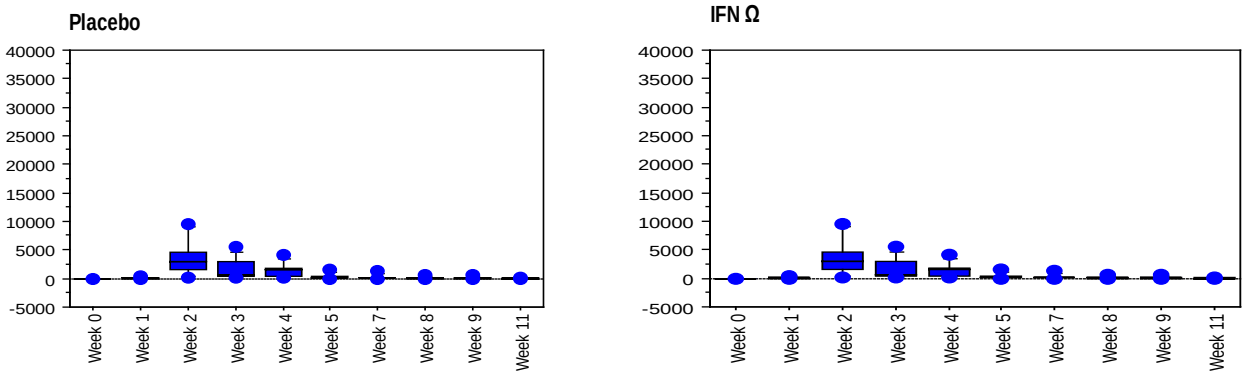
Fig. 19: Proviral load measurements during the experiment

4.3.3. Viral load in plasma:

The viral load in plasma was determined by real- time RT-PCR and calculated as viral copies (10^3/5µl, fig. 20). The RNA copies in plasma showed a short peak starting with week 2 and ending in week 5 for both groups. At no single time point a significant difference between the two values was found.



Frequency distribution:



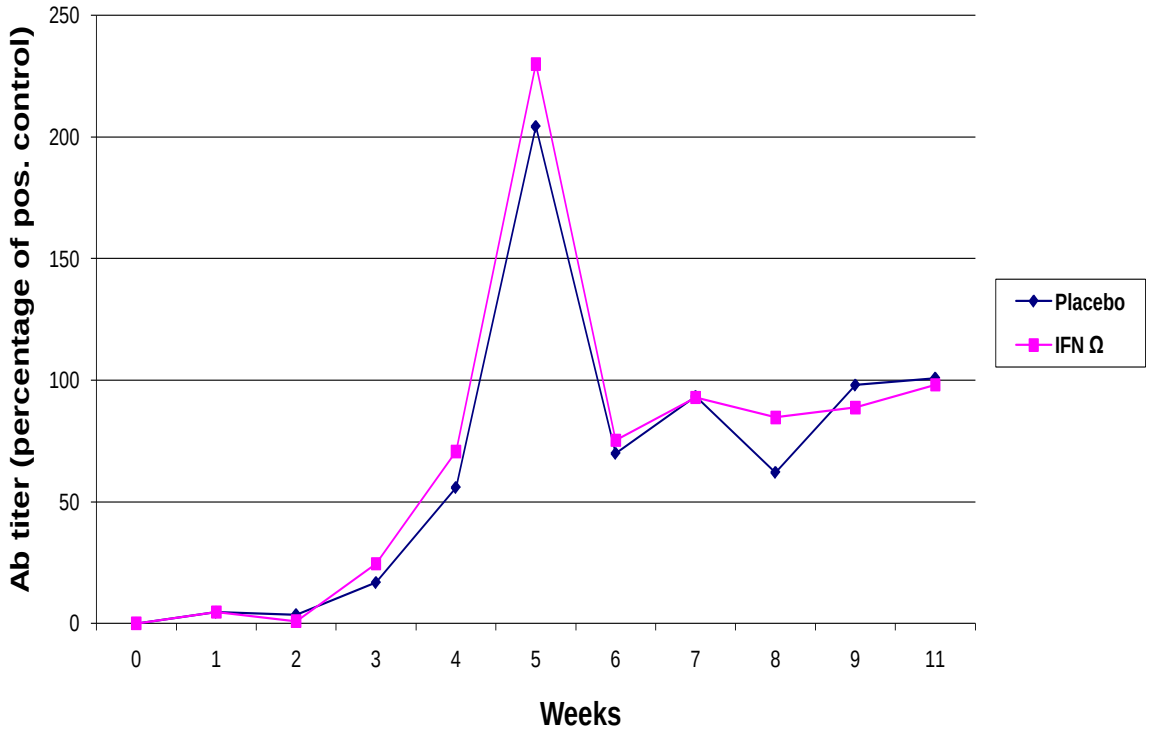
Significance between groups (p-values):

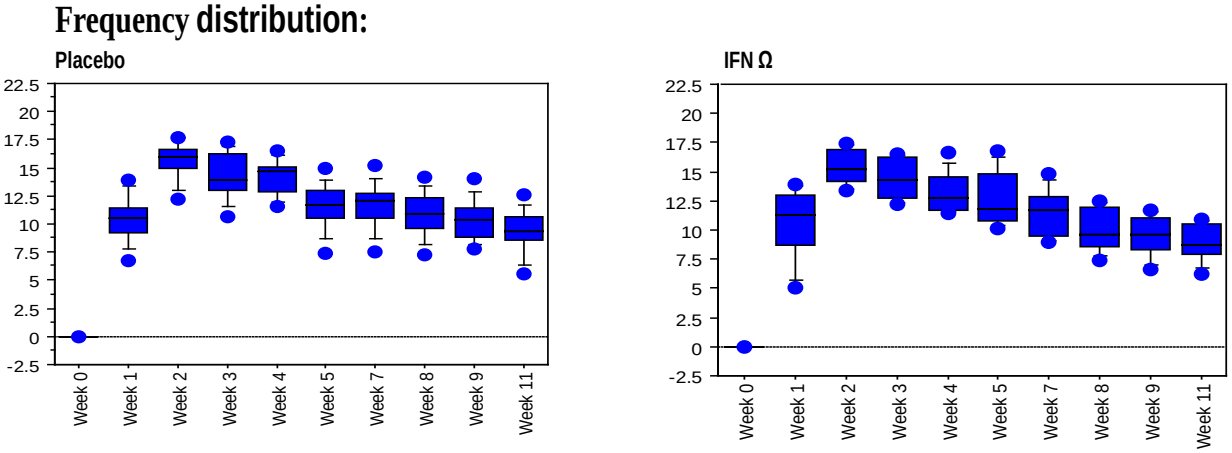
WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

Figure 20: viral load copies in plasma during the experiment

4.3.4. Detection of antibodies to FIV TM by ELISA:

Antibodies to FIV were determined by a TM ELISA for which the results are shown in fig. 21. It becomes evident that antibody development starts already in week 3 with a peak in week 5 for both groups. Seroconversion was identical for both groups displaying only a significant value in week 8.





Significance between groups (p-values):

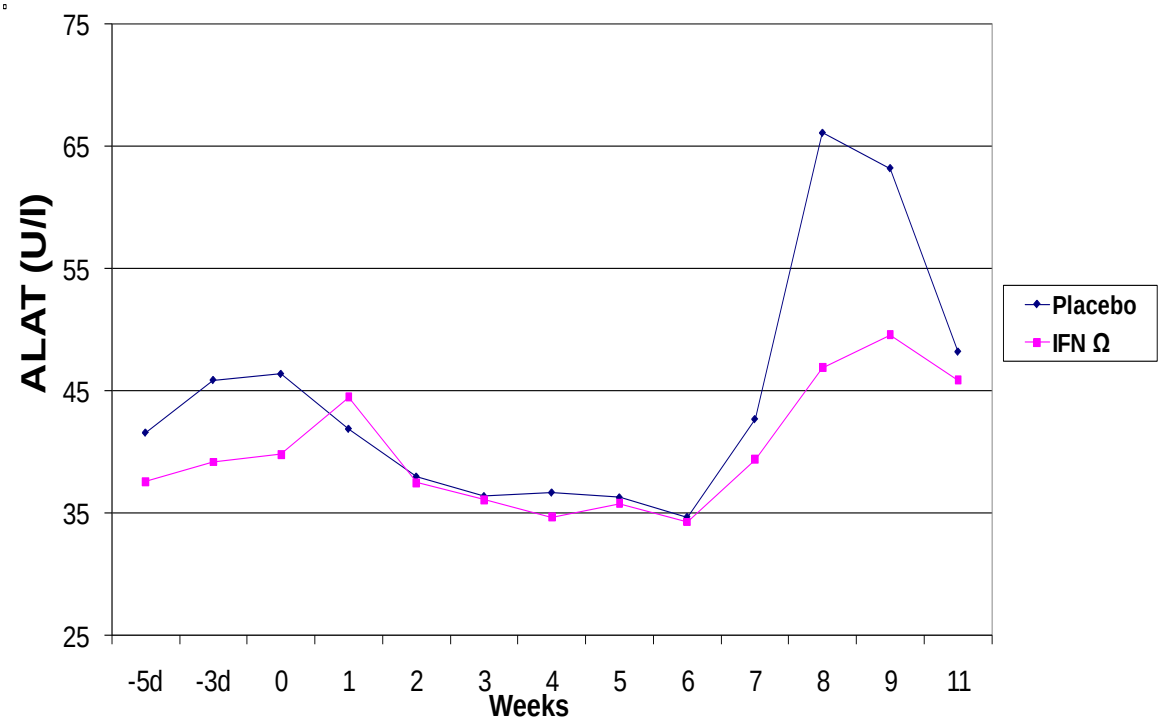
Weeks	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω											0.0305	0.0736	

Fig. 21: Detection of antibodies to FIV TM ELISA during the experiment

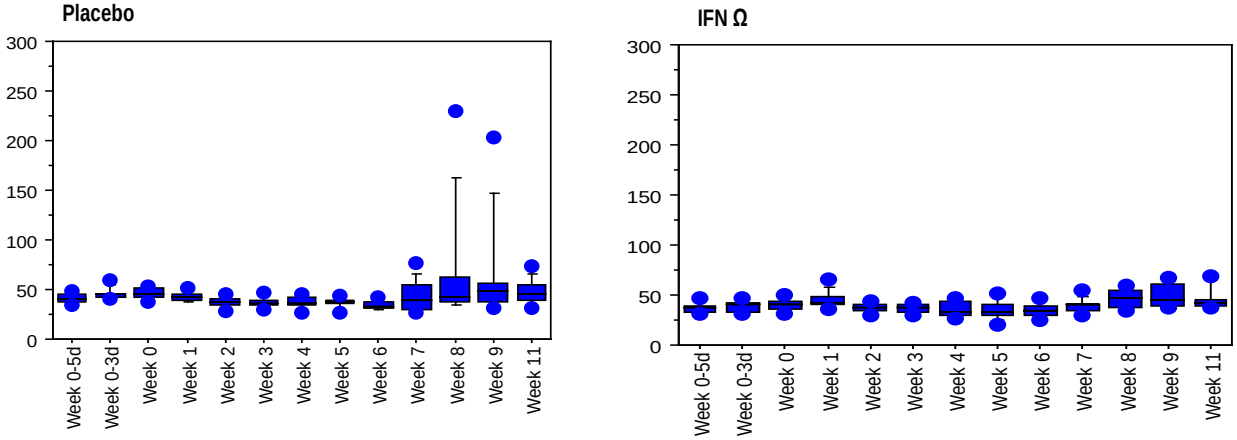
4.3.5. Clinical chemistry values:

4.3.5.1. ALAT:

The ALAT values, which are considered specific for liver affection in the cat species, remained in the normal value range throughout the observation period (fig. 22). Although the values *per se* remained normal there were two time points with significant differences (week 0 –3d, week 0). No explanation can be offered for this difference.



Frequency distribution:



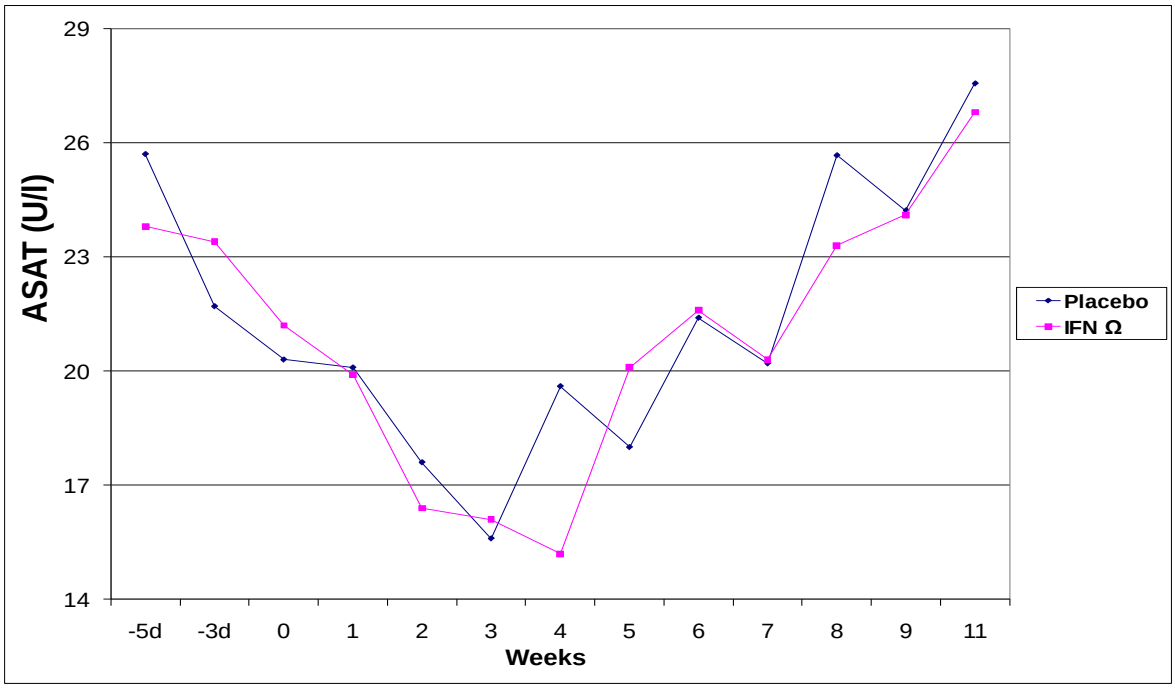
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0156	0.0193										

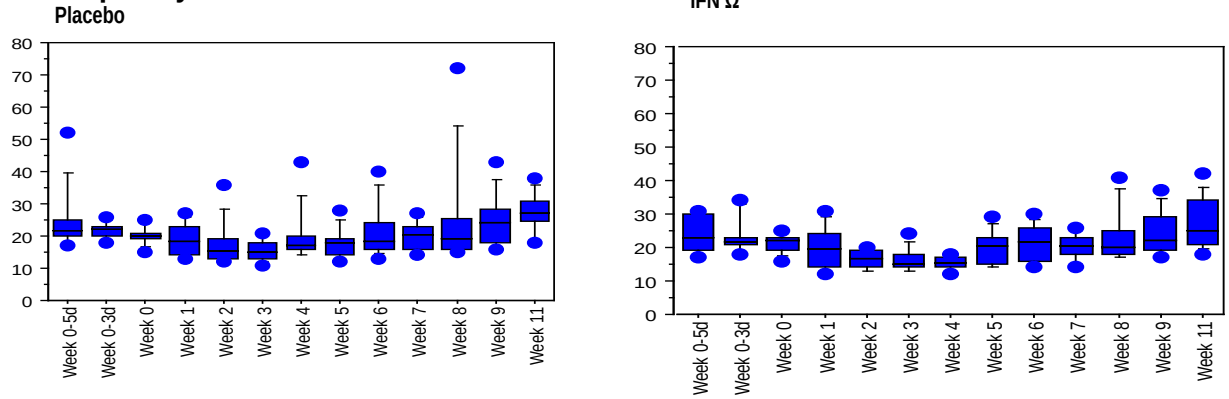
Fig. 22: ALAT values during the experiment

4.3.5.2. ASAT:

The ASAT values remained in the normal reference range throughout the observation period (fig. 22). They dropped to a minimum in the range of 15 U/ L at 3 and 4 weeks of the experiment and later slightly increased again. Mean values of the two groups never showed significant differences.



Frequency distribution:



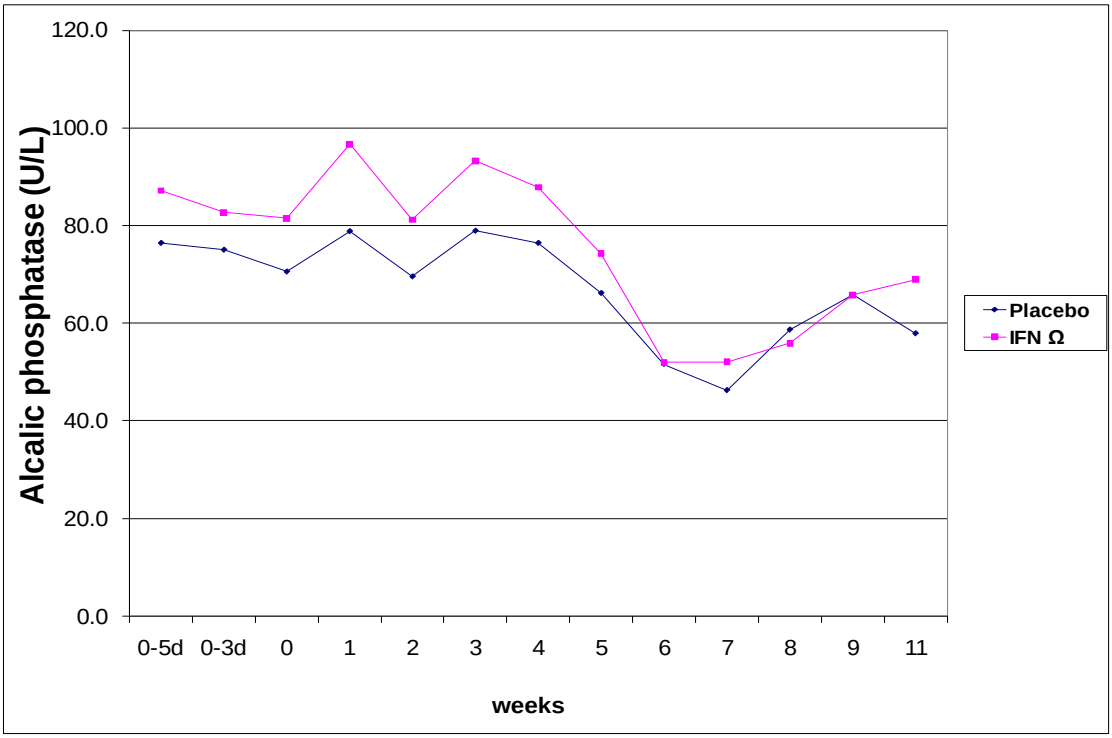
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	W9	w11
Placebo / IFN Ω													

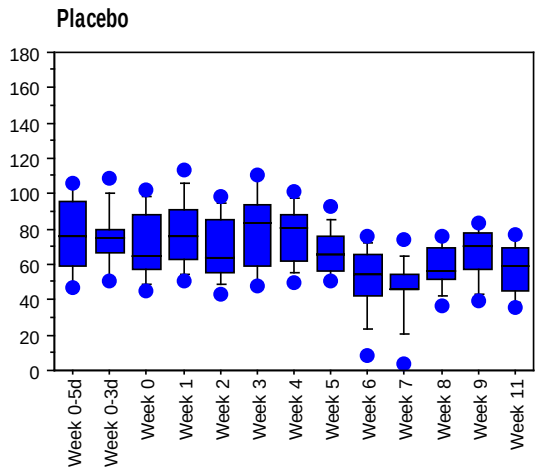
Fig. 23: ASAT values during the experiment

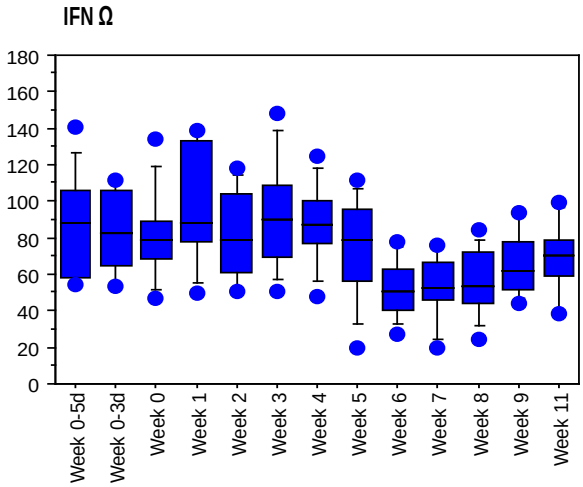
4.3.5.3. Alcaline phosphatase (AP):

The AP values showed almost no variation and no significant differences between the two groups throughout the observation period (fig. 24).



Frequency distribution:





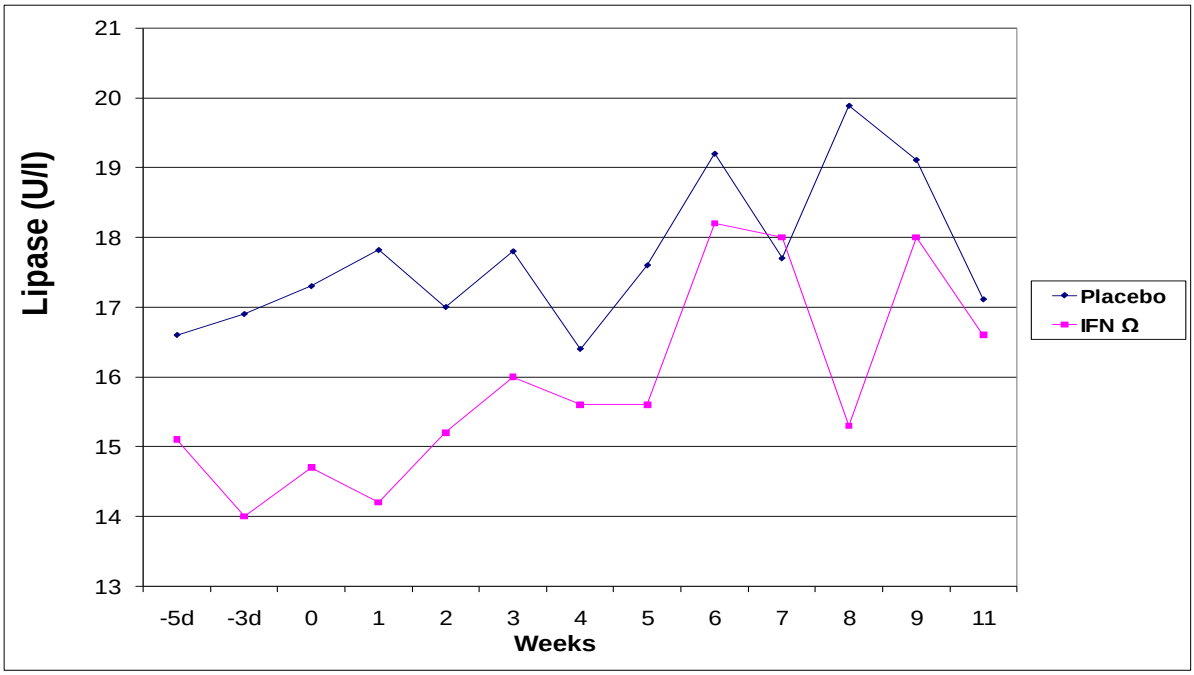
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

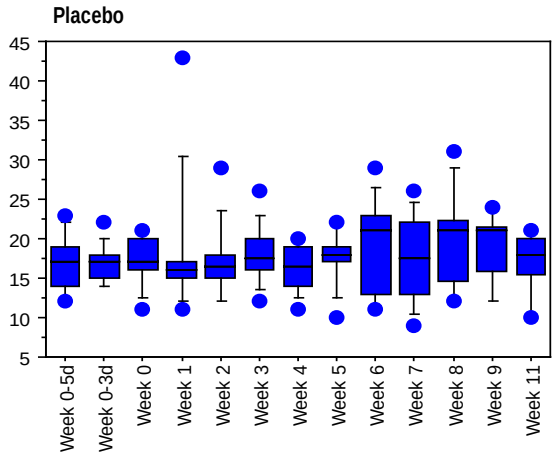
Fig. 24: AP values during the experiment

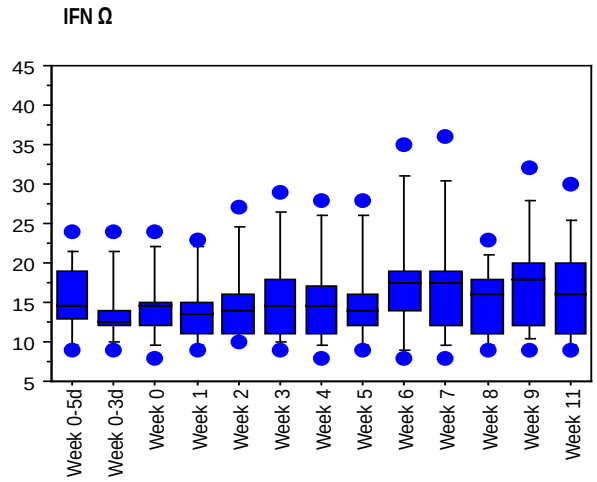
4.3.5.4. Lipase:

Lipase values remained within the normal reference range for cats throughout the entire observation period (fig. 25). Interestingly, lipase values of the IFN Ω group were consistently lower than those in placebo group. The difference was not significant between the two groups at any time point.



Frequency distribution:





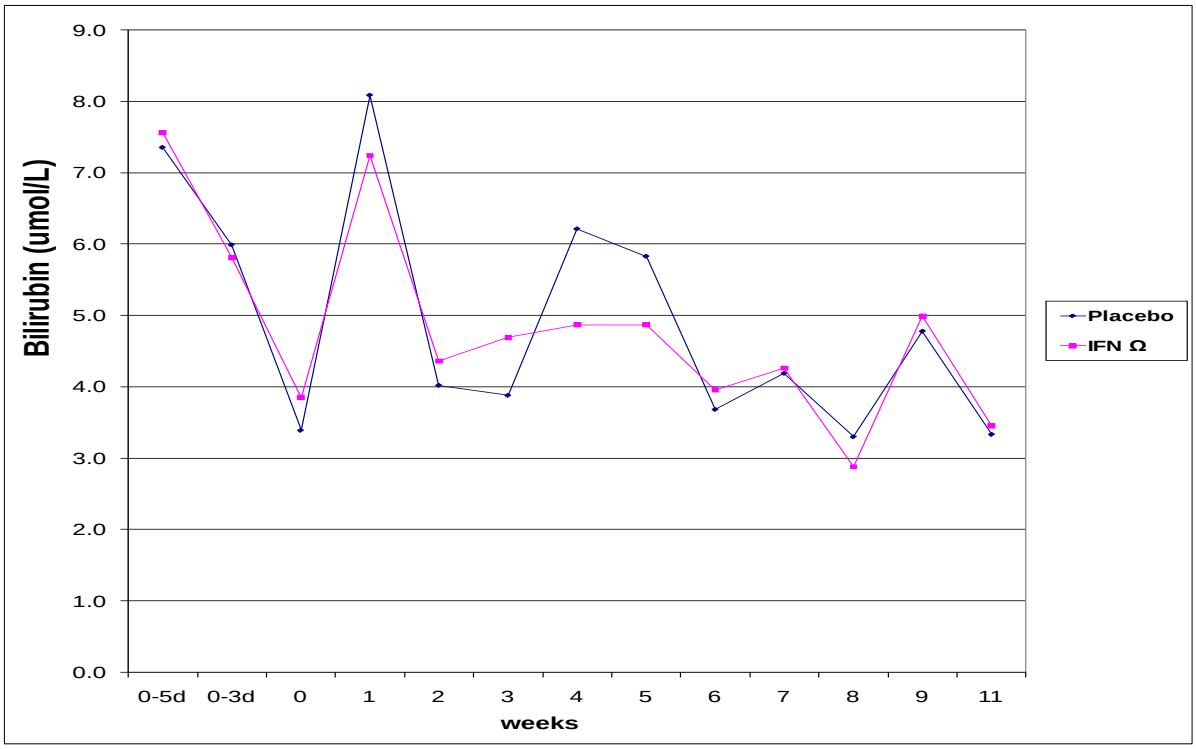
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0806									0.0754		

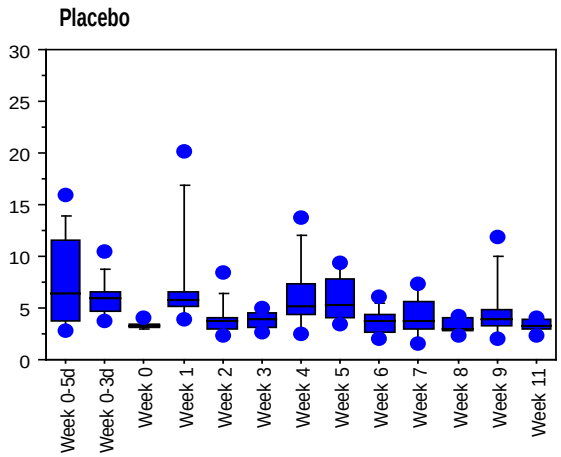
Fig. 25: Lipase values during the experiment

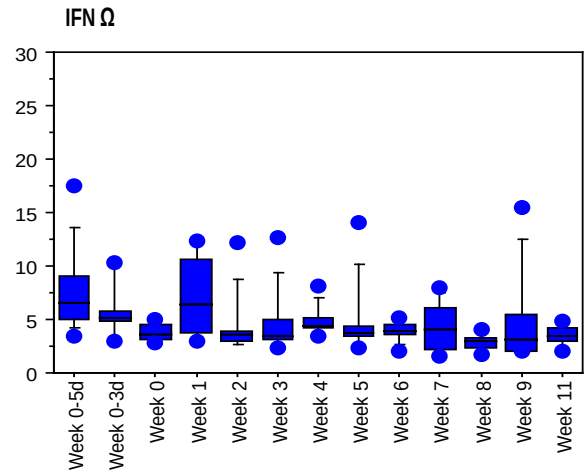
4.3.5.5. Bilirubin:

Bilirubin values remained low and within the reference range throughout the observation period (fig. 26). No significant differences between mean bilirubin values were observed.



Frequency distribution:





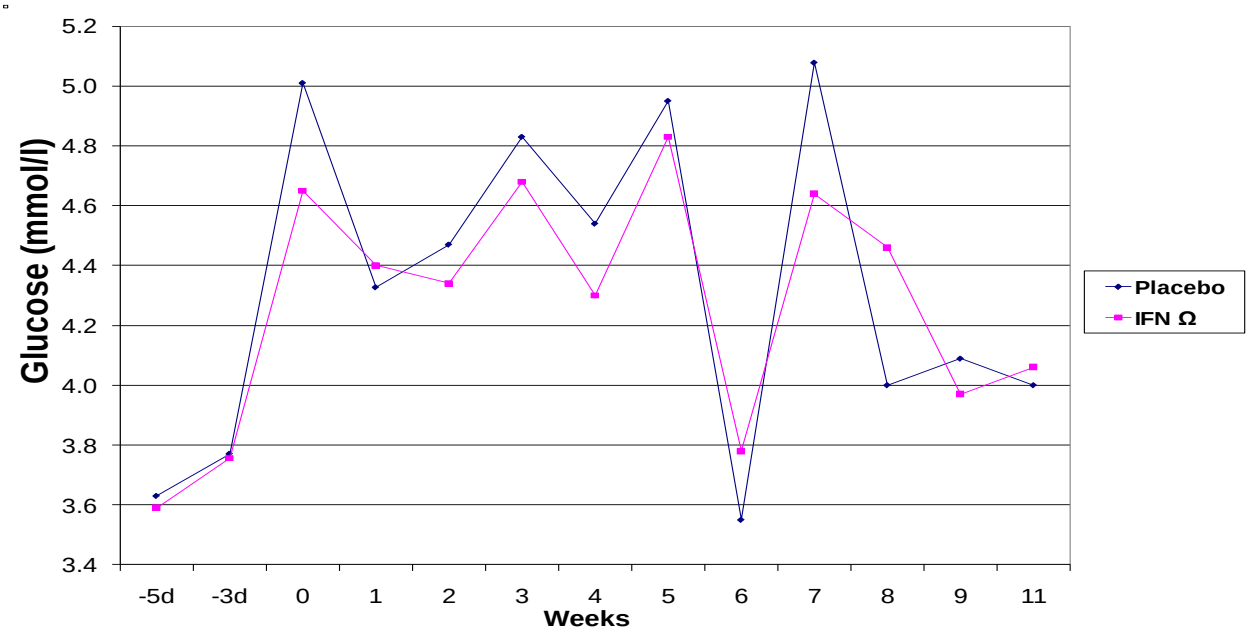
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

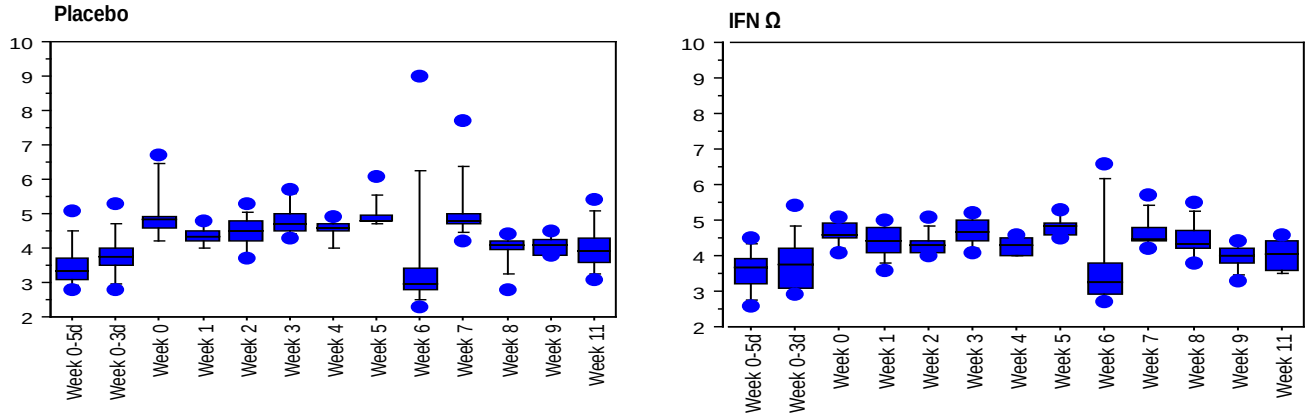
Fig. 26: Bilirubin values during the experiment

4.3.5.6. Glucose:

Glucose values were always in the normal reference range throughout the observation period (fig. 27). They never showed significant differences between the two groups, however, all glucose values were in the lower range of the reference interval. This is explained by the fact that the cats were fastened before blood collection. The low values had nothing to do with the treatment and/or FIV infection. All values in week 6 were in the lower range of the reference interval most likely due to the fact of an extended storage period because the analyser was not functioning in time.



Frequency distribution:



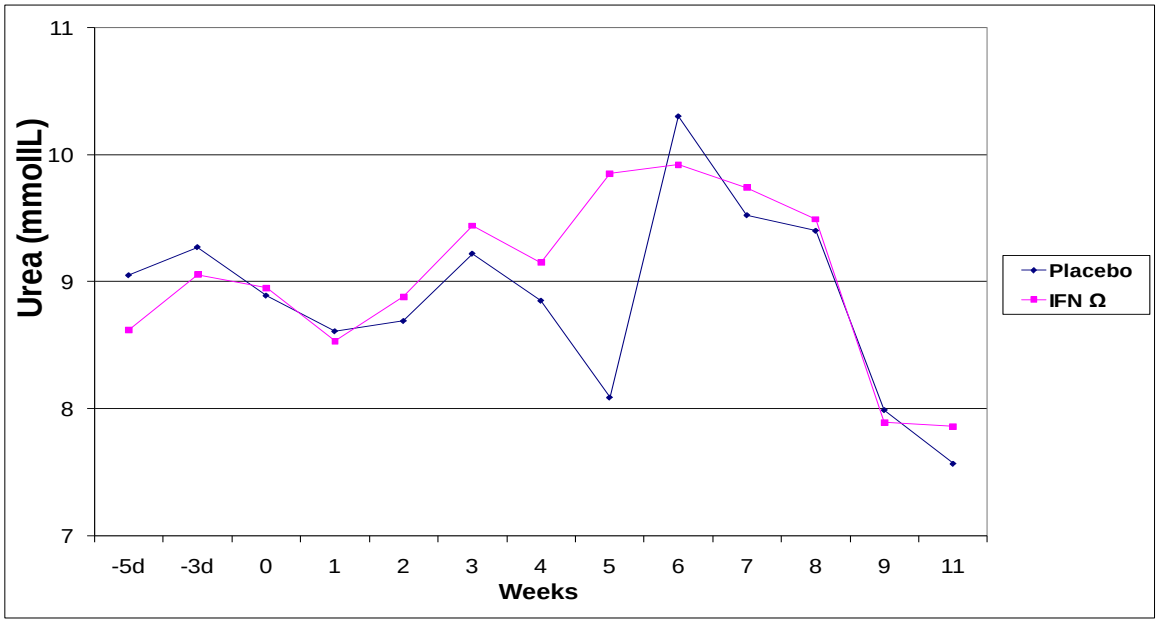
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω							0.0688				0.0575		

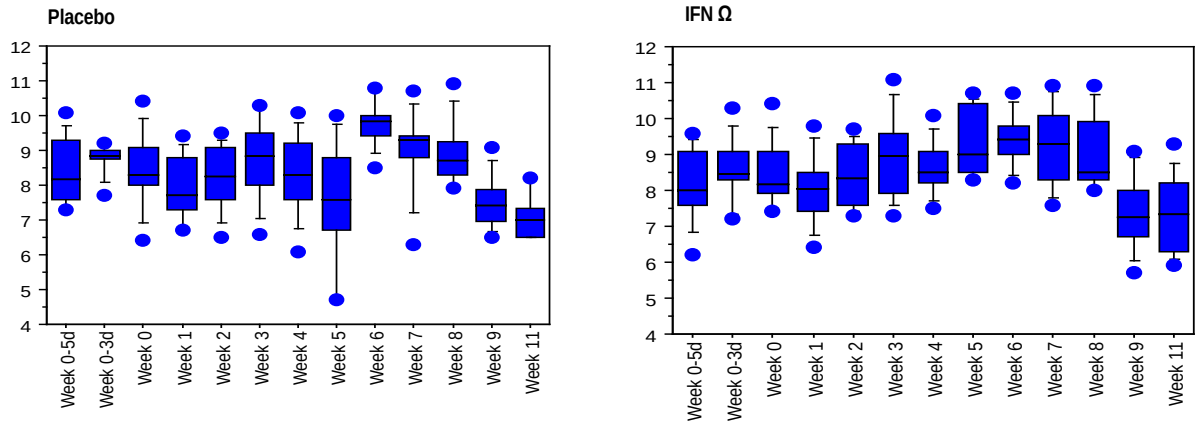
Fig. 27: Glucose values during the experiment

4.3.5.7. Urea:

In fig. 28 the course of the urea levels in the two groups is displayed. Again, with the exception of week 5, no significant differences were observed. No explanation for the differences in week 5 can be offered. However, is is speculated that the cats of the IFN Ω group had received more food than those of the placebo group resulting in a lower production of urea in the placebo group.



Frequency distribution:



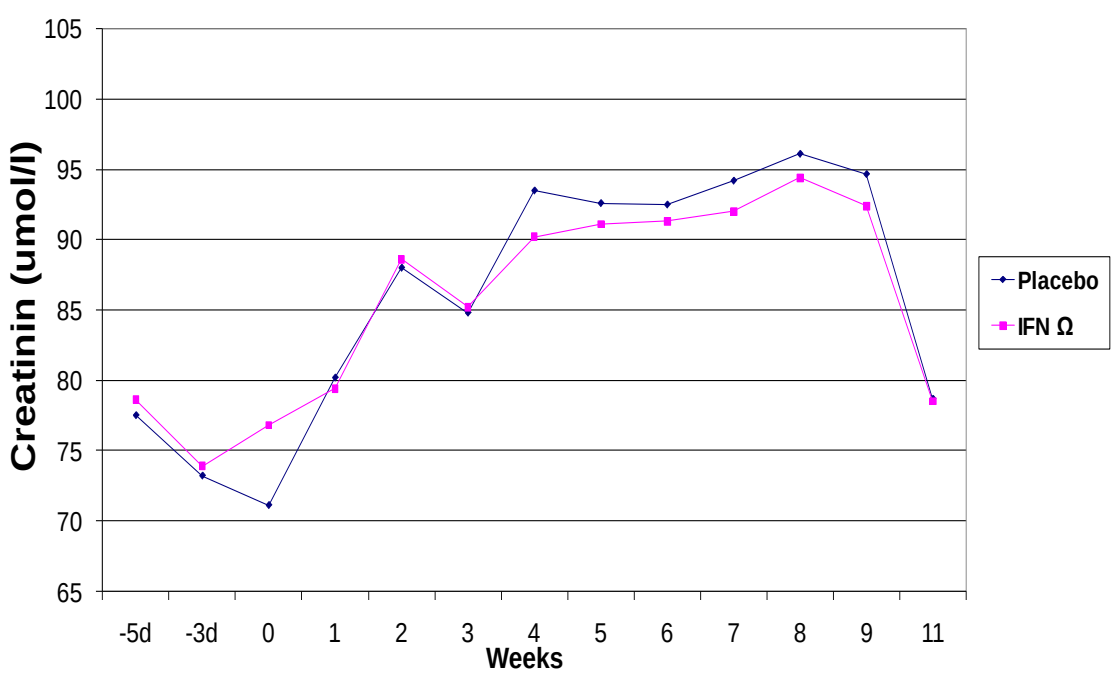
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω								0.0131					

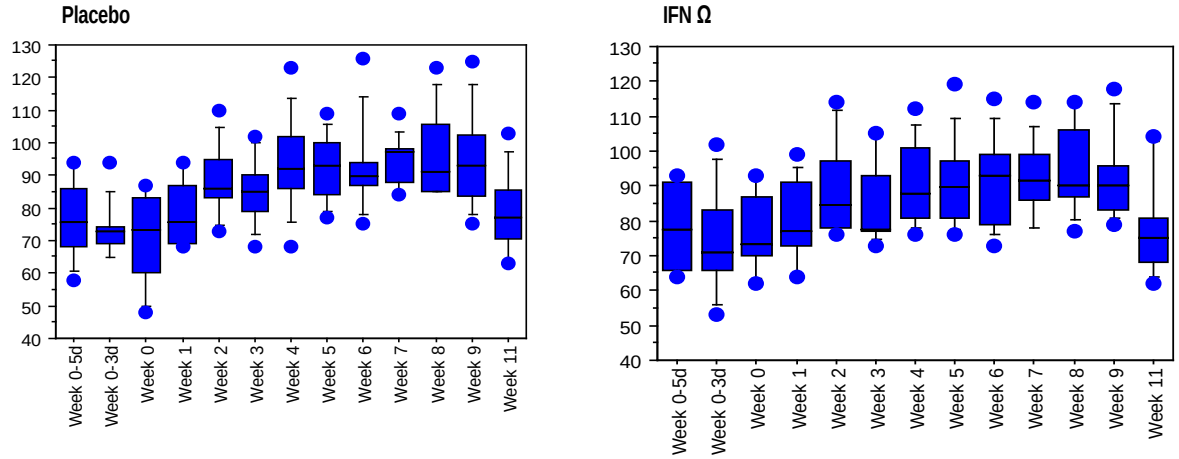
Fig. 28: Blood urea levels during the experiment

4.3.5.8. Creatinin:

The creatinin values rose steadily over the duration of the observation period (fig. 29). This increase most likely reflects the muscle mass, which increased parallel to the growth of the cats. The last value in week 11 dropped dramatically in both groups; no explanation can be offered.



Frequency distribution:



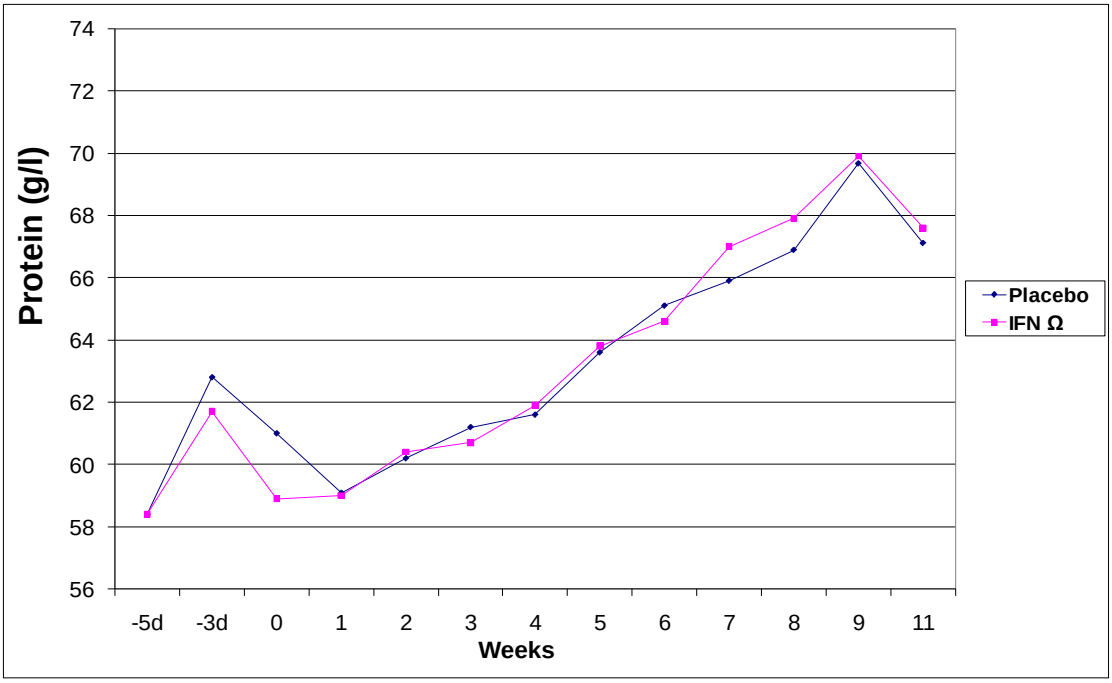
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	W2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

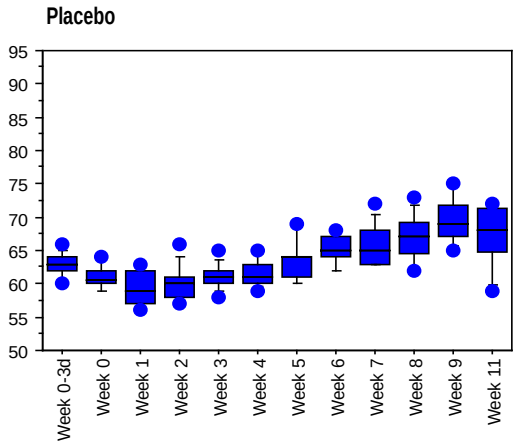
Fig. 29: Blood creatinin values during the experiment

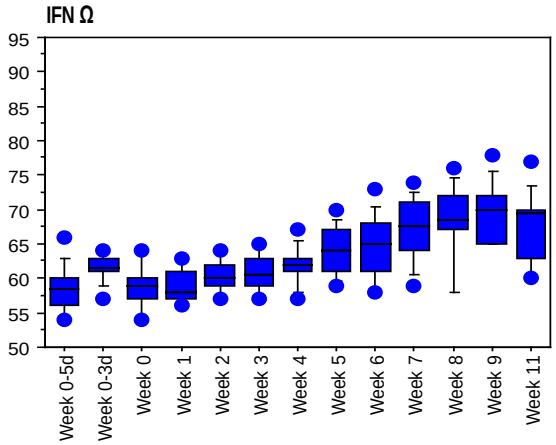
4.3.5.9. Protein concentration:

As shown for the creatinin values, the concentration of the serum protein steadily increased over the duration of the observation period (fig. 30). There were no statistically significant differences between the groups at any time point.



Frequency distribution:-





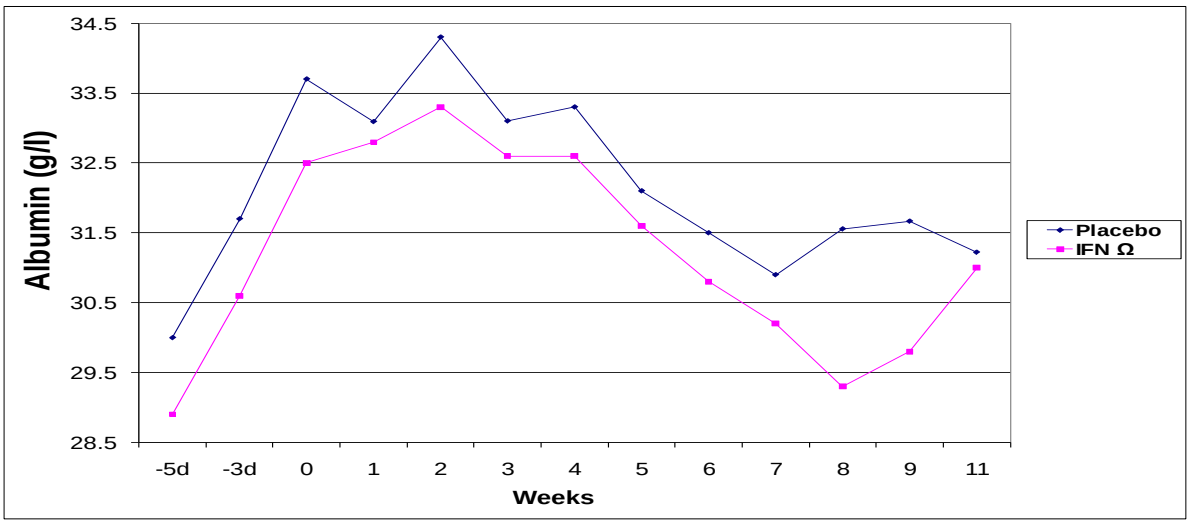
significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

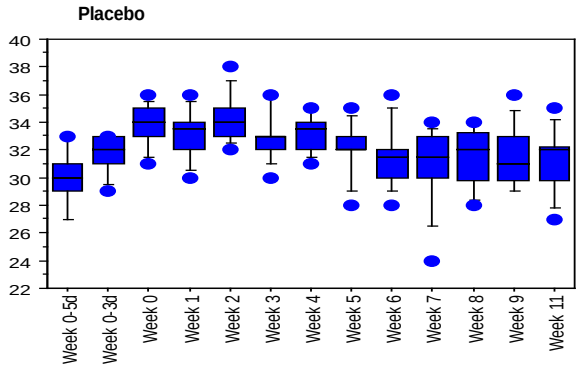
Fig. 30: Protein concentration values during the experiment

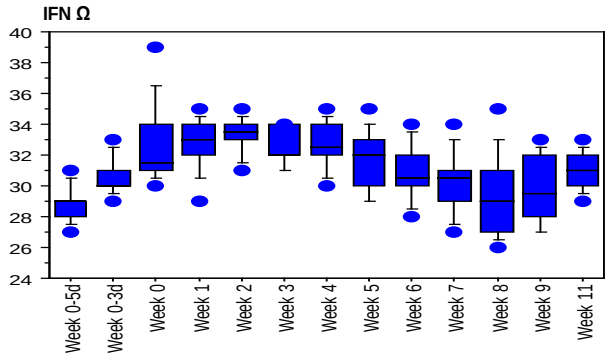
4.3.5.10. Albumin:

In fig. 29, the albumin concentrations measured in serum are displayed. The albumin concentrations were systematically lower in animals of IFN Ω group, although there was no significant difference at any one point. Interestingly, over the first three weeks of observation, the albumin values rose in both groups, remained roughly at the same level until week 4 and then systematically dropped. Although no explanation for this decrease can be offered it is striking that it parallels seroconversion to FIV TM and with that the immune reaction against FIV. The difference between total protein and albumin is globulin, and the drop in albumin shown in fig. 31 is inversely parallel to the increase of globulin during the same time. Therefore, the drop of albumin values is most likely due to the FIV infection occurring similarly in both groups.



Frequency distribution:





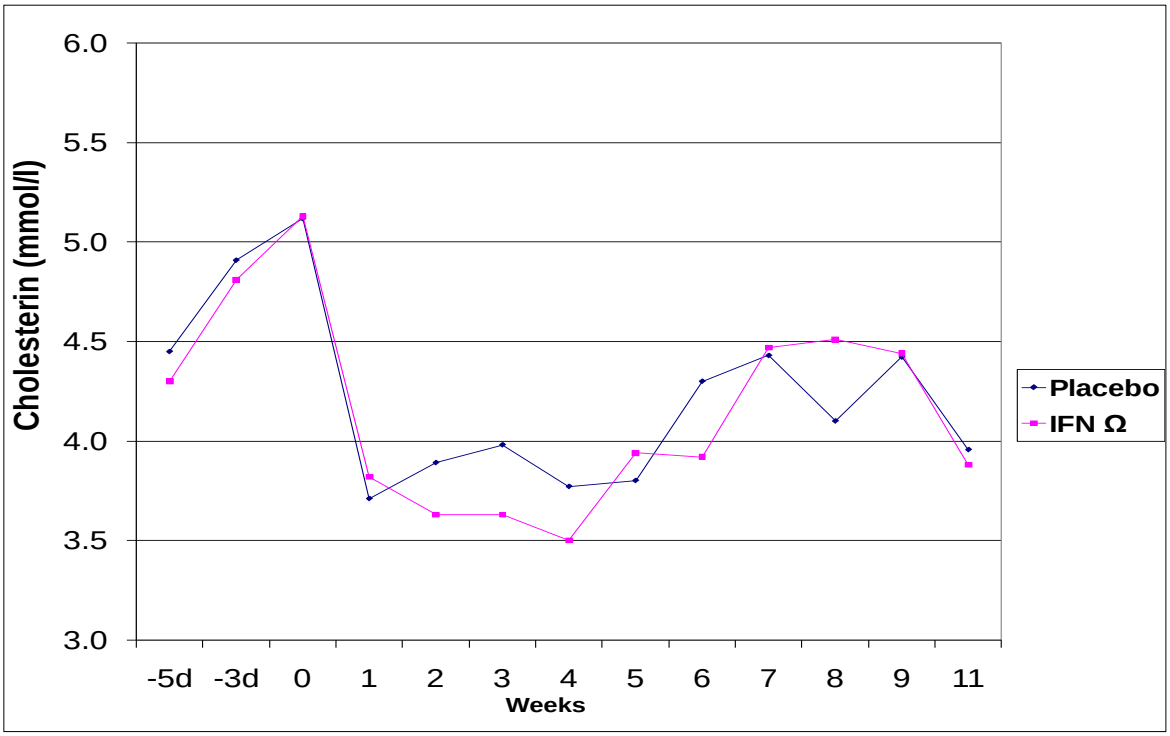
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0663									0.0571		

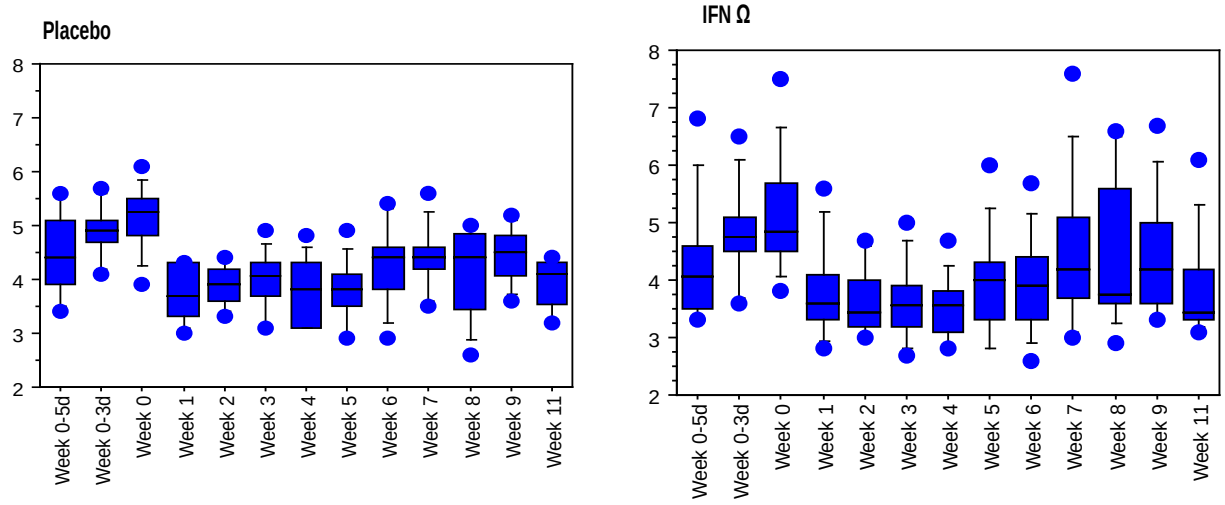
Fig. 31: Albumin concentrations during the experiment

4.3.5.11. Colesterol:

The levels of colesterol remained within the normal range throughout the observation period (fig. 32). No difference between the two groups was seen at anyone time point.



Frequency distribution:



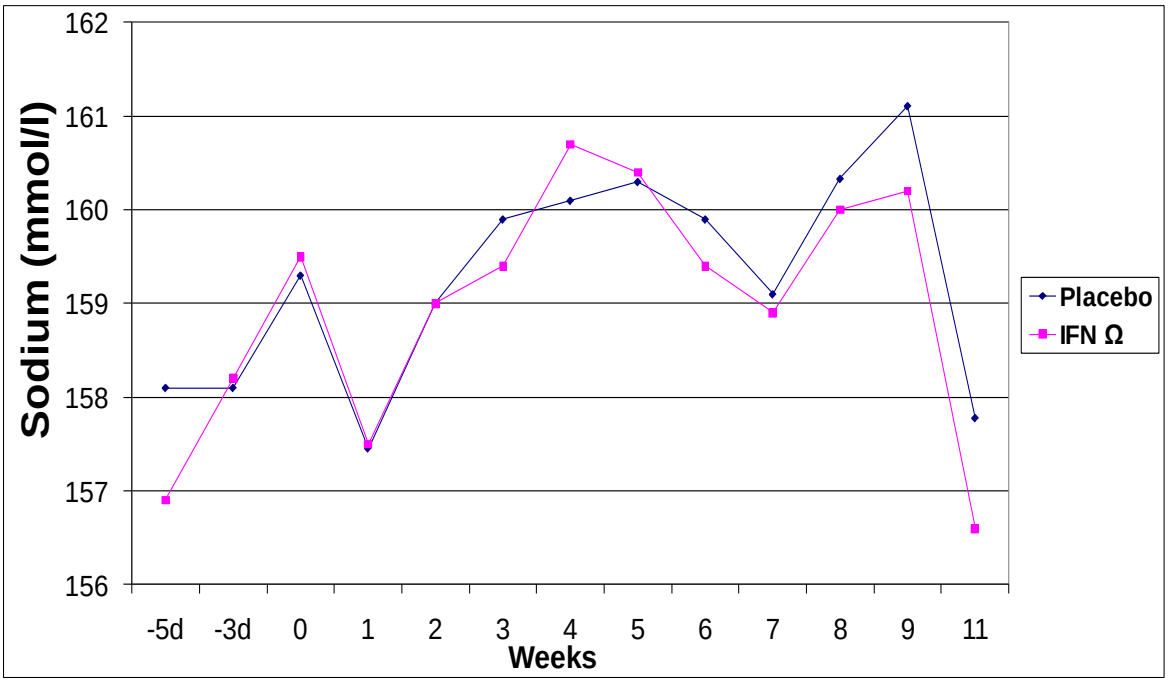
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	W2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

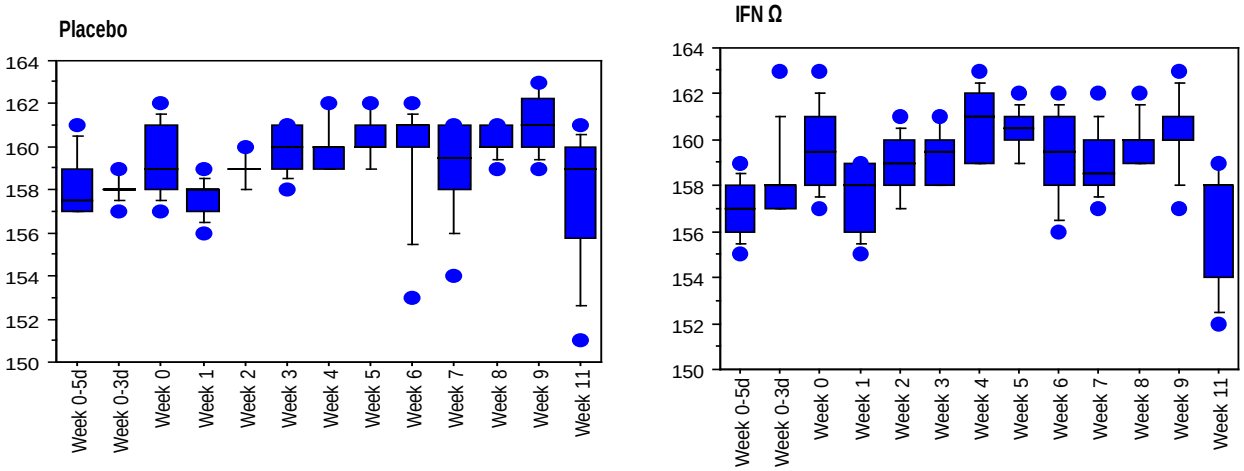
Fig. 32: blood colesterin levels during the experiment

4.3.5.12. Sodium:

Development of sodium concentrations is shown in fig. 33. With the exception of the very first blood collection in week 0 –5d, no significant differences were observed.



Frequency distribution:



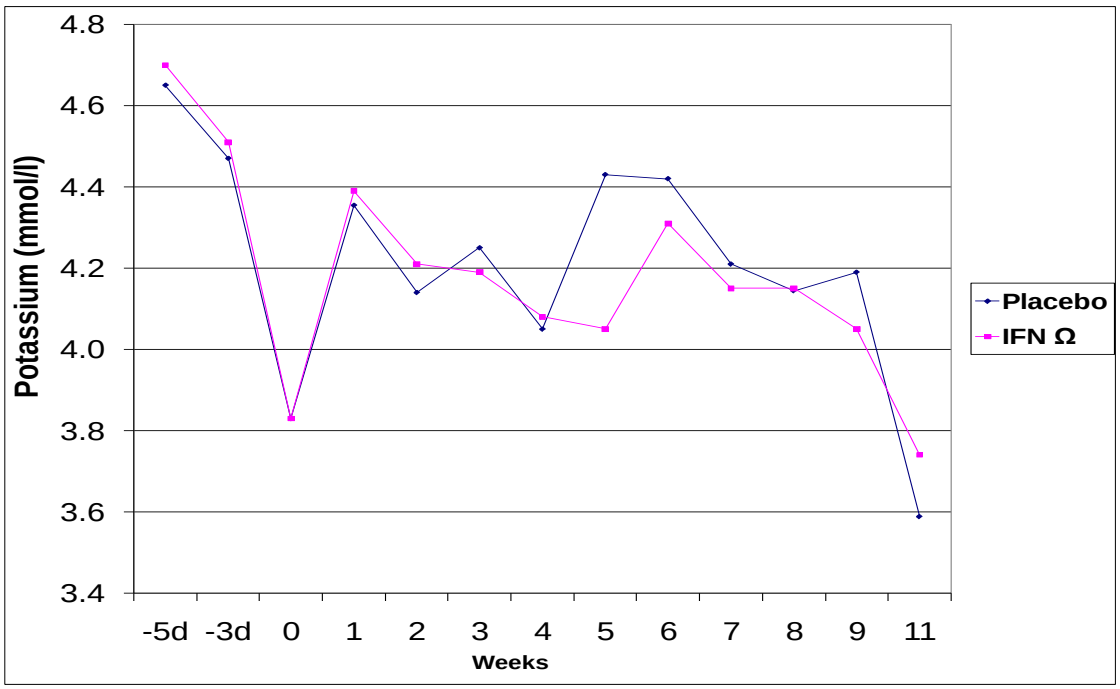
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω	0.0442												

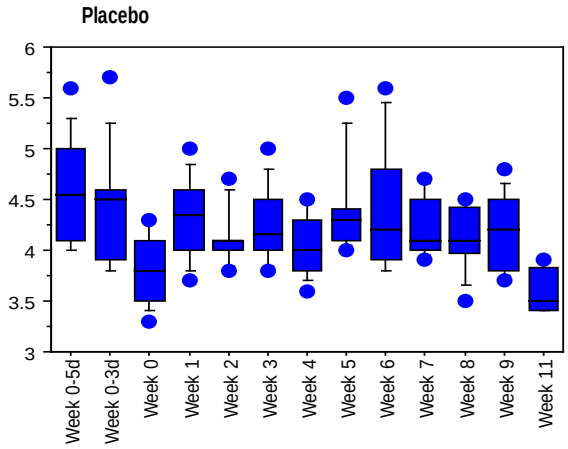
Fig. 33: Sodium concentrations during the experiment

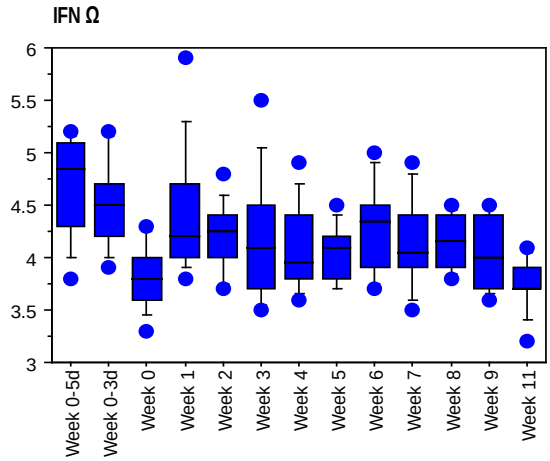
4.3.5.13. Potassium:

The course of the potassium concentrations is shown in fig. 34. Overall, in both groups the potassium concentration steadily decreased with increasing duration of the observation period. At one time point (week 5) there was a significant difference between the potassium values in both groups; no explanation for this difference can be offered.



Frequency distribution:





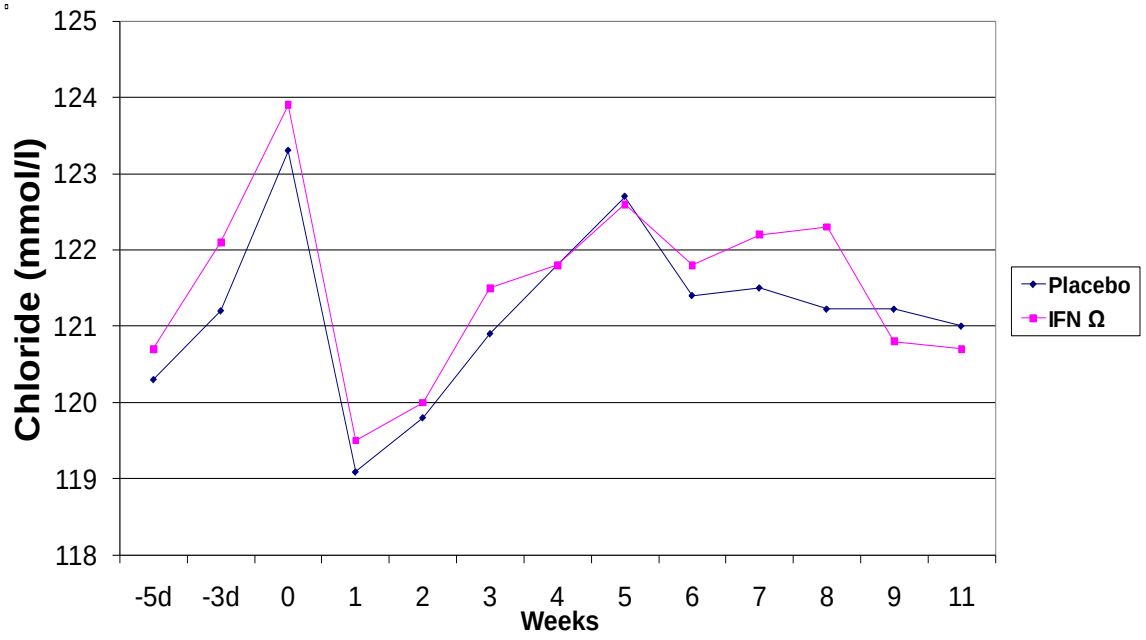
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω								0.0385					

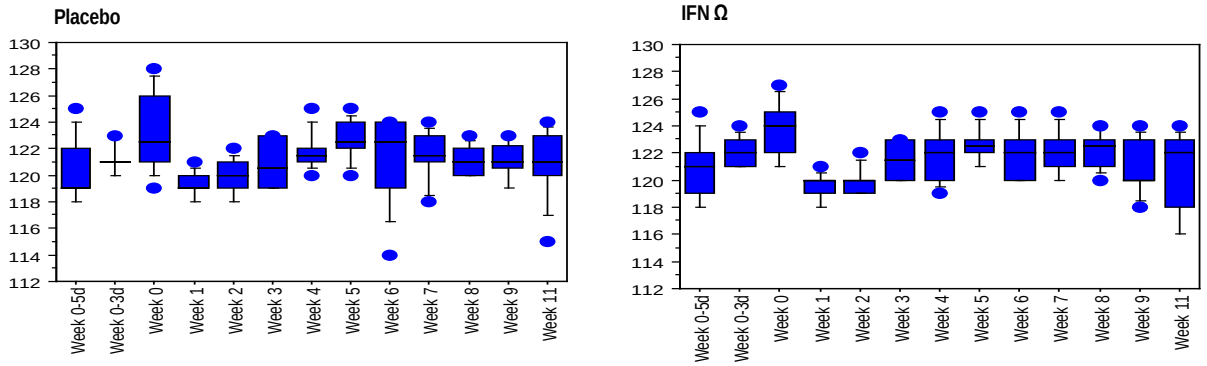
Fig. 34: Blood potassium concentrations during the experiment

4.3.5.14. Chloride:

The course of the chloride values of both groups resembled that of the sodium values displaying an increase for the first three collections followed by a strong drop in week 1 and then again followed by an increase. The drop in week 1 coincides with the first week of FIV infection; however, no explanation for this drop can be offered (fig. 35). Again, as before, no significant differences between the values of the two groups could be observed.



Frequency distribution:



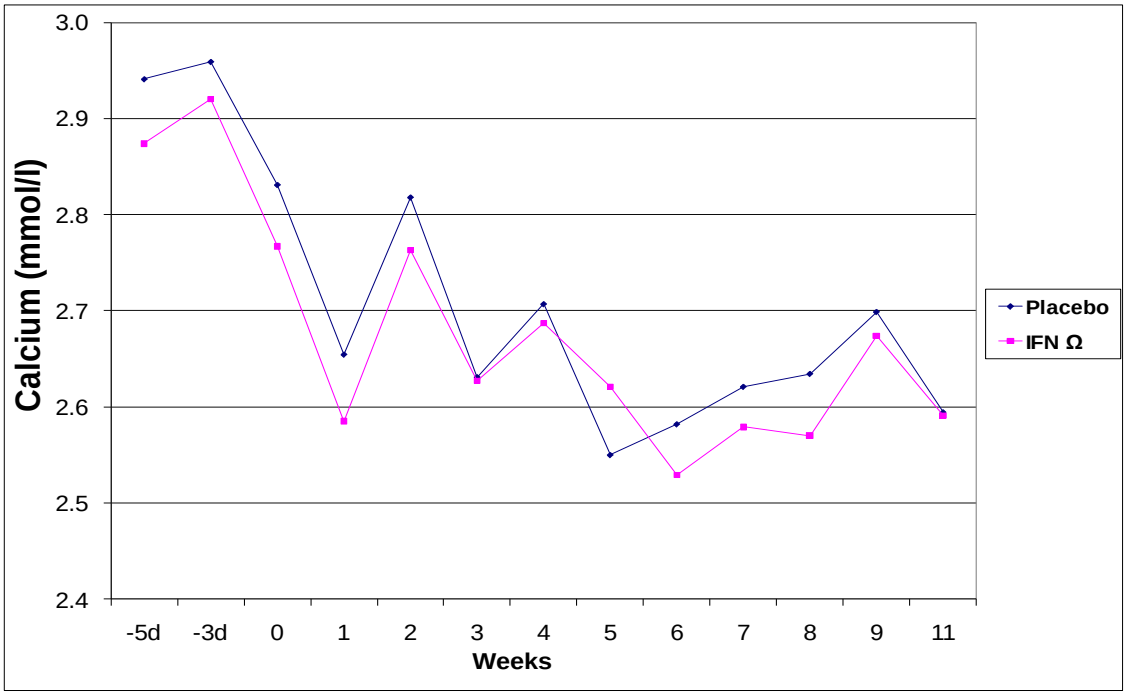
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω		0.0626									0.0732		

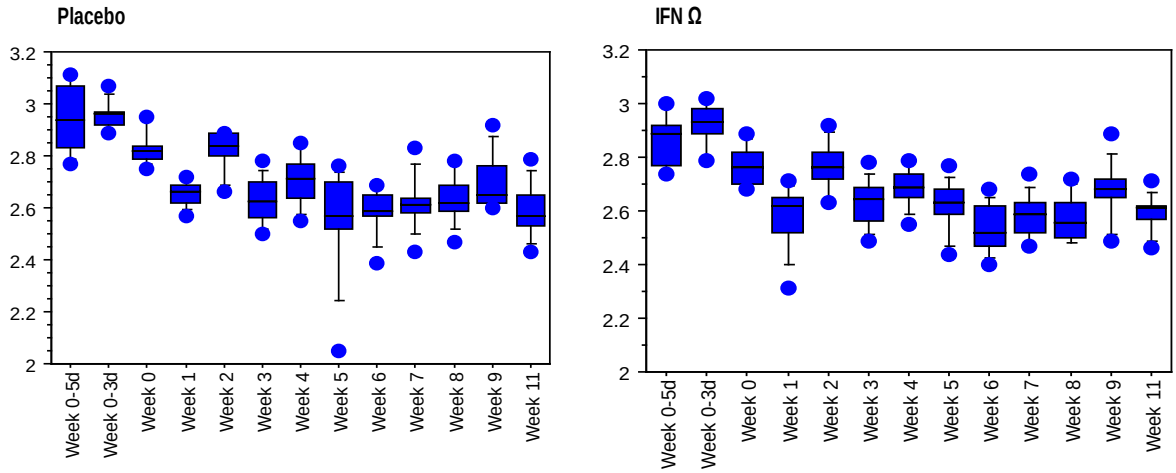
Fig. 35: Blood chloride values during the experiment

4.3.5.15. Calcium:

The calcium concentration steadily declined with increasing time of observation (fig. 36). There was a significant difference between the mean values of the two groups at week 0. Otherwise, the values in both groups followed a parallel course. No explanation can be offered for the drop by week one other than that by this time infection had started.



Frequency distribution:



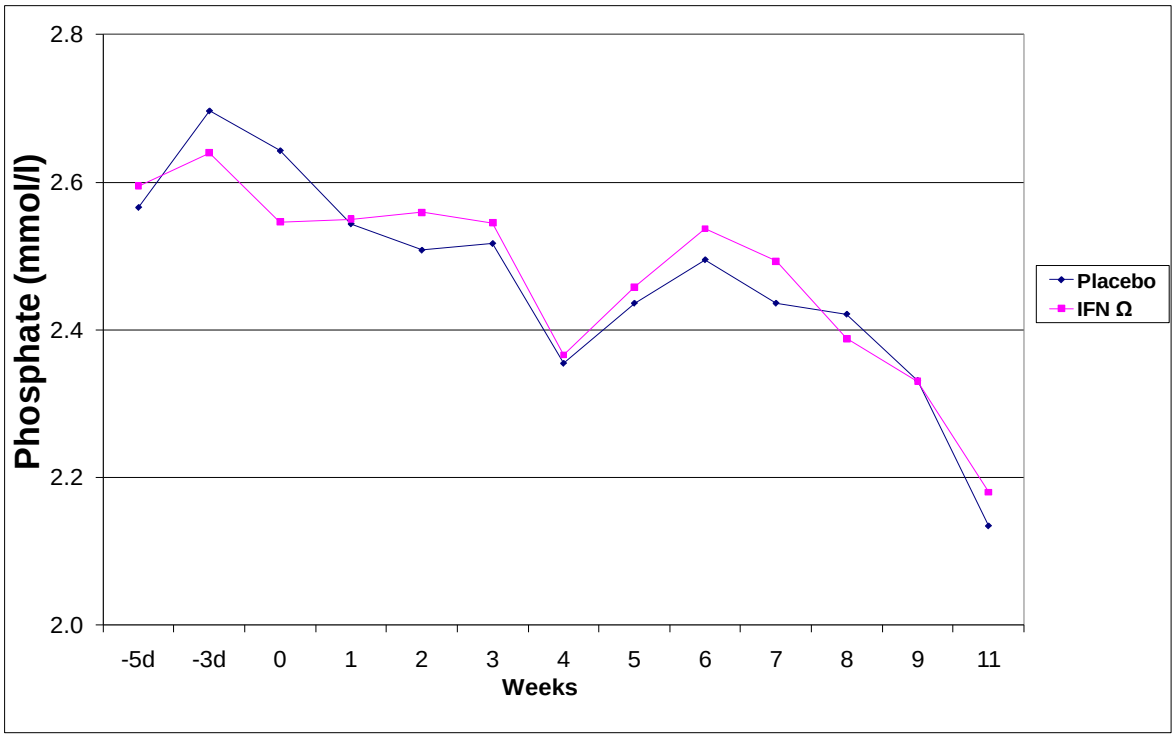
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω			0.0446										

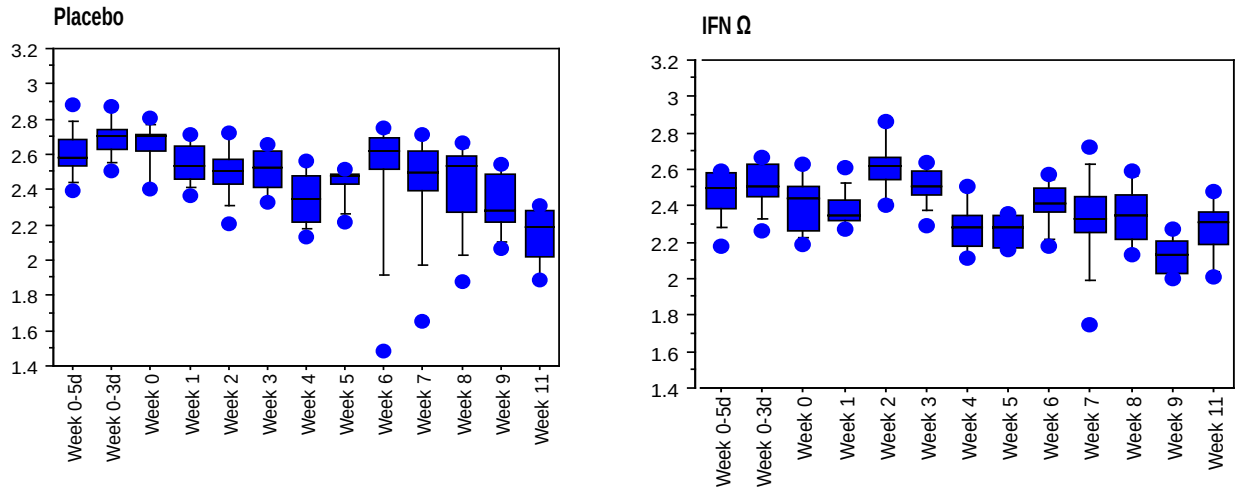
Fig. 36: Calcium concentrations during the experiment

4.3.5.16. Phosphate:

The phosphate values declined steadily over the duration of the observation period (fig. 37). No significant differences between the two groups at anyone of the time points were observed.



Frequency distribution:



Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

Fig. 37: Blood phosphate values during the experiment

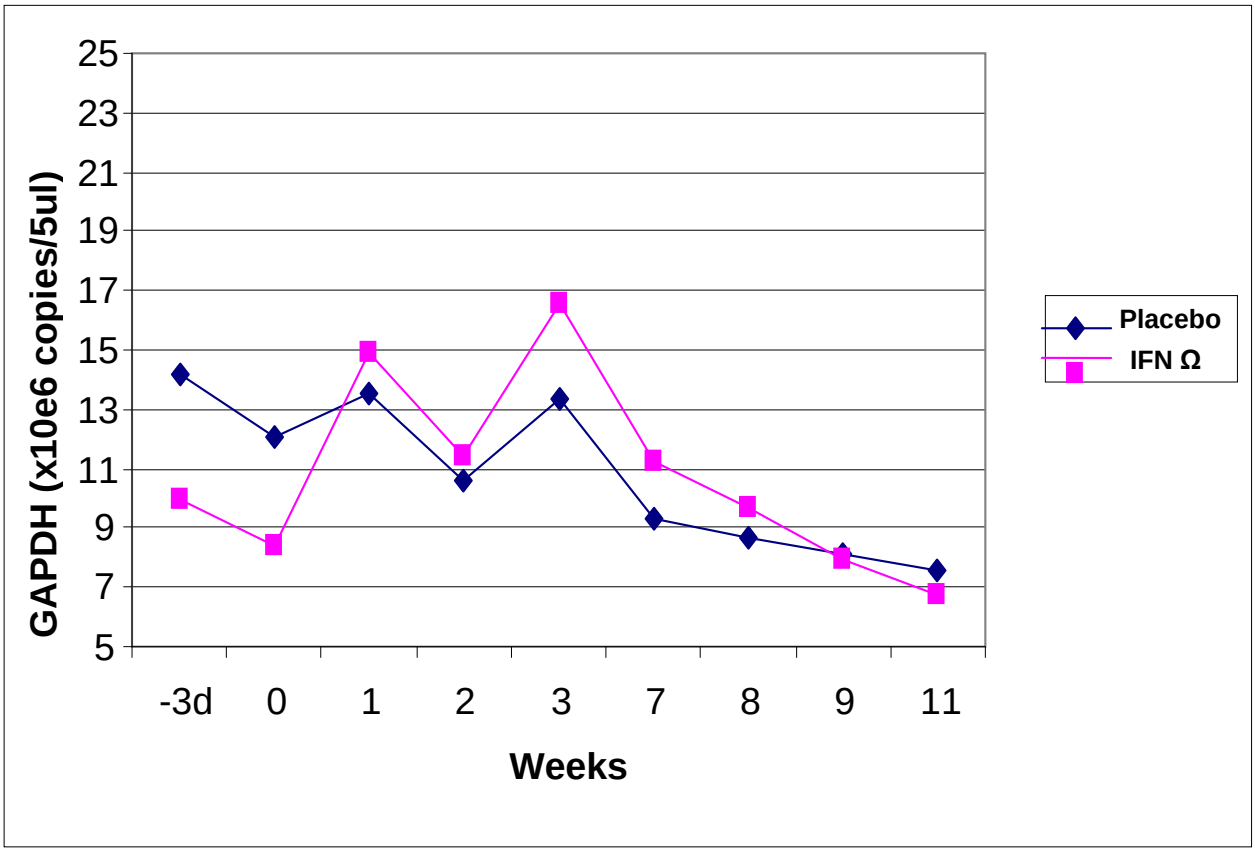
4.4. Measurement of cytokine expression:

4.4.1. Stability of mRNA in purified form:

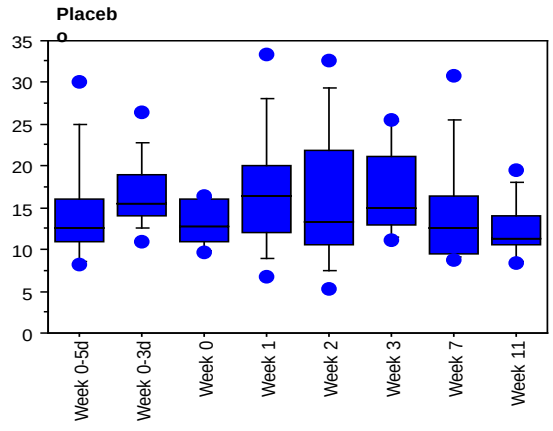
mRNA was purified from the blood samples immediately after collection of blood and stored at –70°C until analysis. As the cytokine measurements could not be done simultaneously, the question had to be answered whether mRNA in the storage tube remained stable even if the tube had to be thawed and refrozen several times. To this end, aliquots were taken from tubes containing purified mRNA, which were thawed and refrozen one to five times with daily intervals. These aliquots were stored at –70°C until all aliquots were assayed for GAPDH together at one occasion. In paragraph 3.5.4 the Ct values of thawed and refrozen samples were determined; the results differed less than 1 Ct value. As no systematic change in the values was observed, we concluded that aliquots of mRNA can be used without loss of mRNA concentration even if the stock sample from which the aliquots had been taken, had been thawed and refrozen several times.

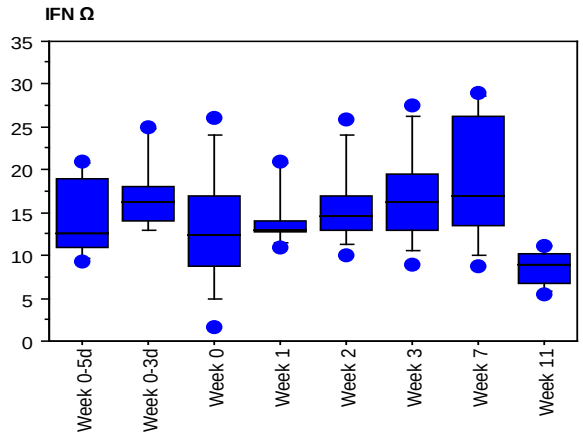
4.4.2. Measurement of GAPDH

Total mRNA was extracted from whole blood (equivalent containing 10^6 WBCs) from which an aliquot was assayed for presence of GAPDH mRNA copies. In fig. 36 the concentration of GAPDH mRNA copies over time is displayed. It becomes evident that the mean GAPDH value is quite similar in both groups and that the variation is minimal. No differences between the GAPDH values in the mean values of the two groups are found with the exception of the final collection where there was a small but significant difference. We concluded from this stability that GAPDH can readily be used as an internal standard to which cytokine expression can be calculated.



Frequency distribution:





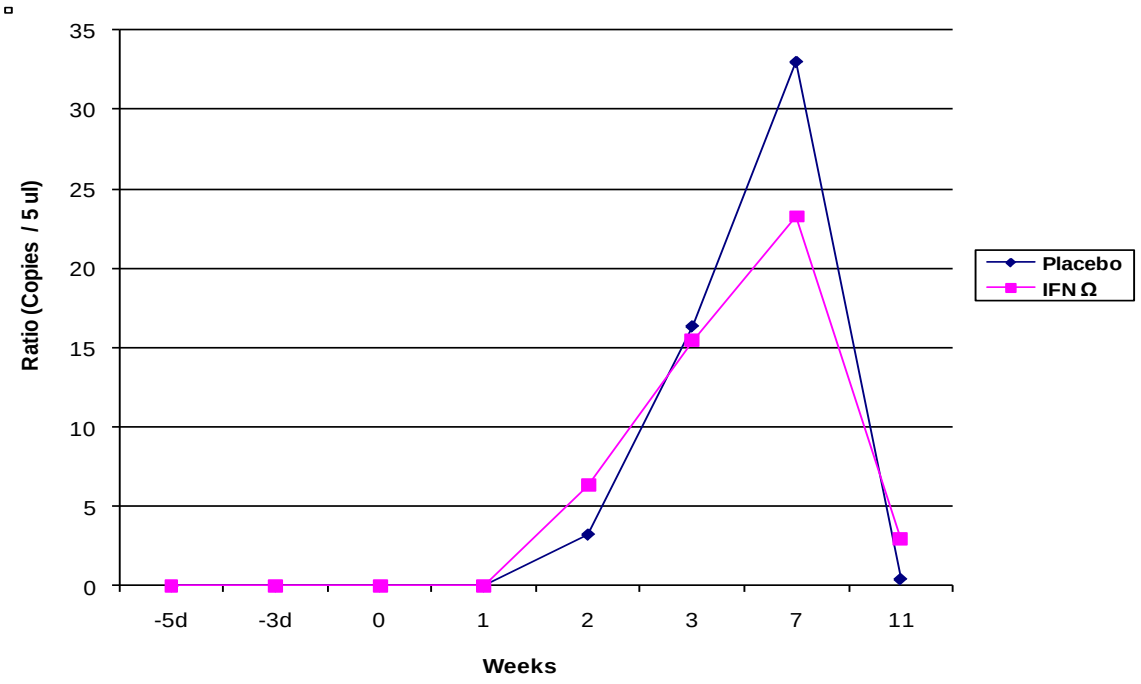
Significance between groups (p-values):

Group	w0-5d	w0-3d	w0	w1	w2	w3	w7	w8	w9	w11
Placebo / IFN Ω	0.0327	0.0570	0.0773							0.0130

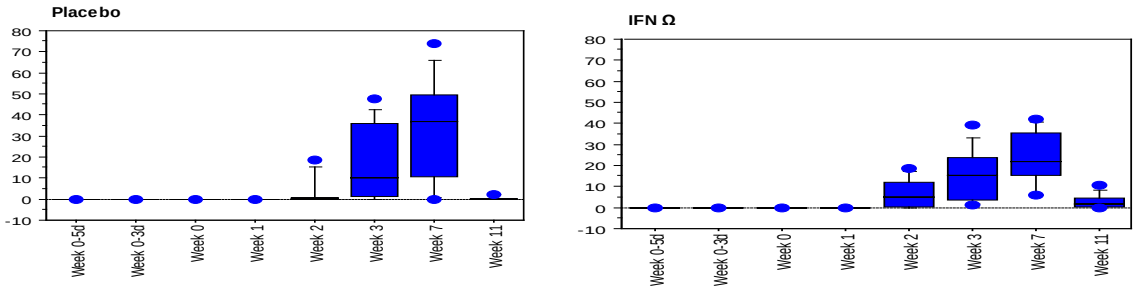
Fig. 38: GAPDH concentrations during the experiment

4.4.3. Ratio of IL-10 to GAPDH:

In fig. 39 the ratio of IL-10 to GAPDH is displayed. It becomes evident that by week 2 after challenge infection this ratio strongly increases until week 7 and then drops by week 11, where a small but significant difference is found.



Frequency distribution:

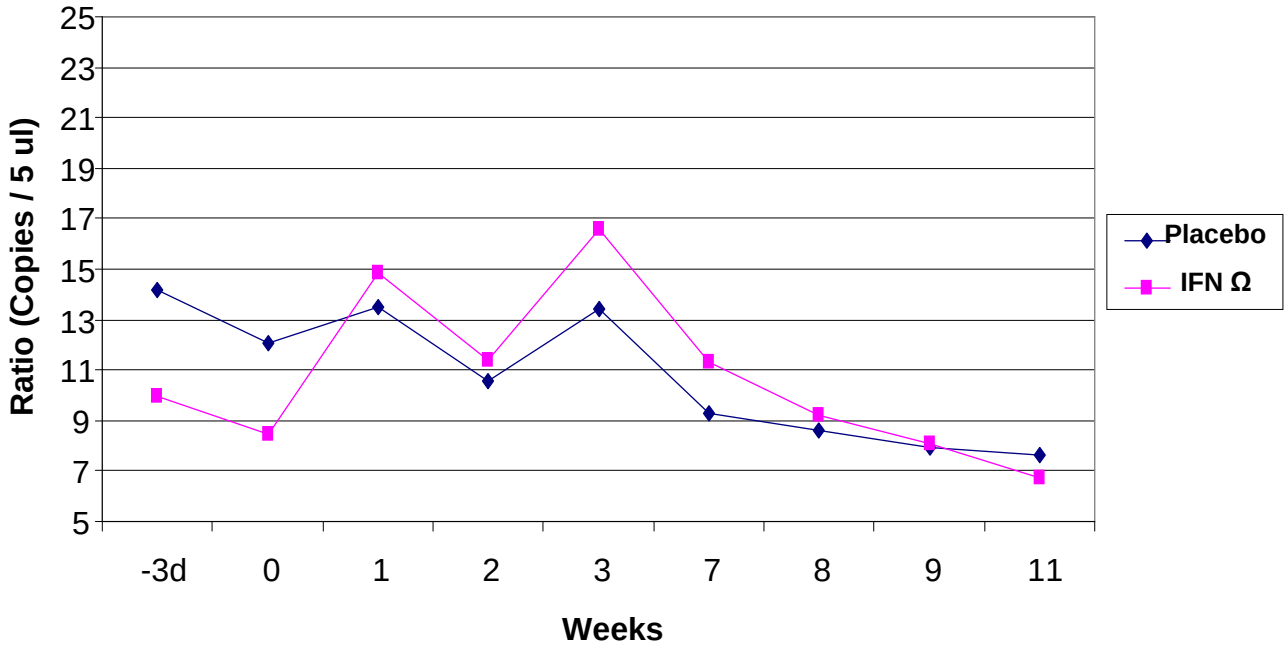


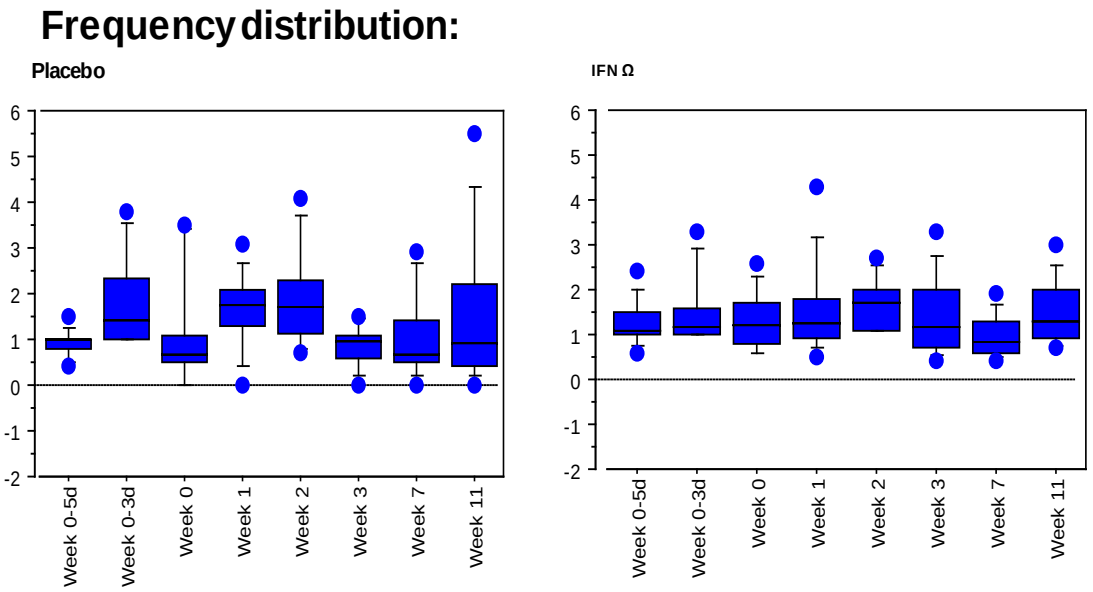
	Weeks												
Groups	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo/ IFN Ω	0.0436	0.032	0.003	0.059					0.048	0.018	0.026	0.031	

Fig. 39: IL -10 to GAPDH ratio during the experiment

4.4.4. IL-12 p40 to GAPDH ratio:

Over the entire observation period the IL-12 p40 to GAPDH ratio remained almost identical with values in the range of 0.8 to 1.7. No time correlation with infection was seen.





Significance between groups (p-values):

Group	wo-5d	w0-3d	w0	w1	w2	w3	w7	w11
Placebo/ IFN Ω	0.0959							

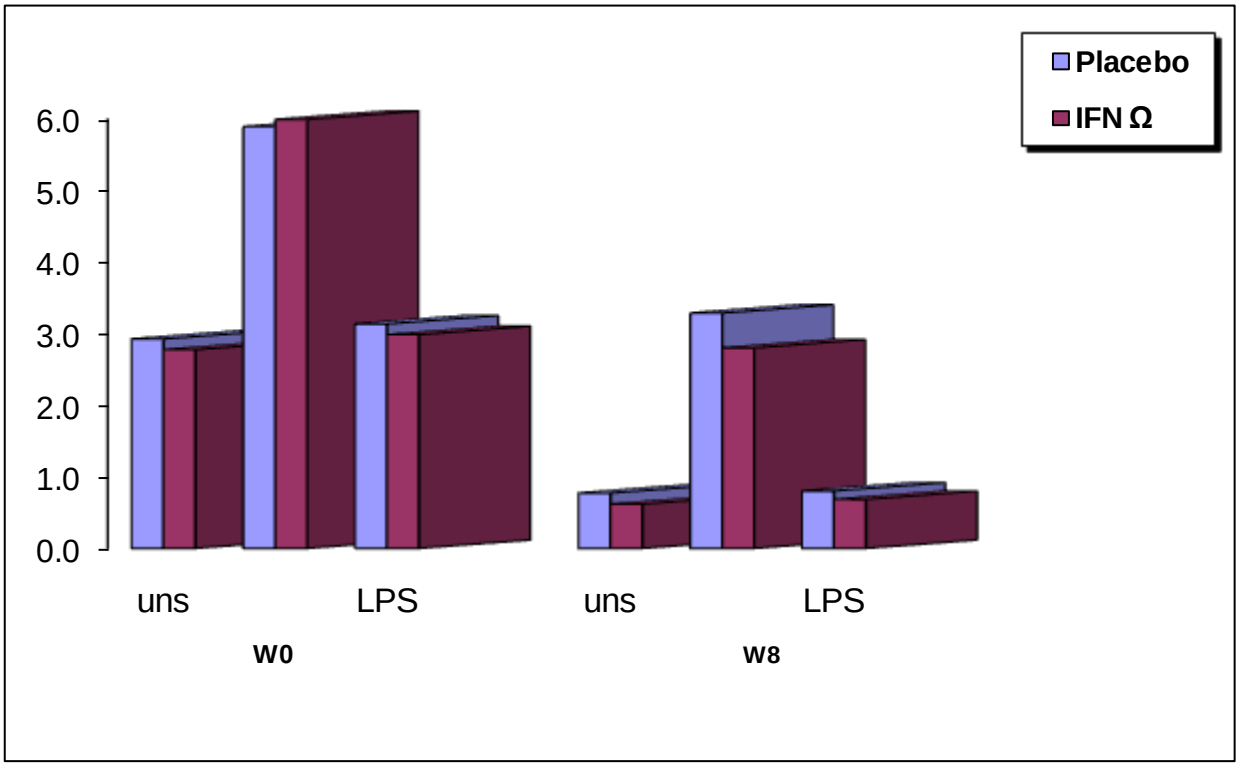
Fig. 40: IL -12 p40 to GAPDH ratio during the experiment

4.4.5. Interferon gamma to GAPDH ratio:

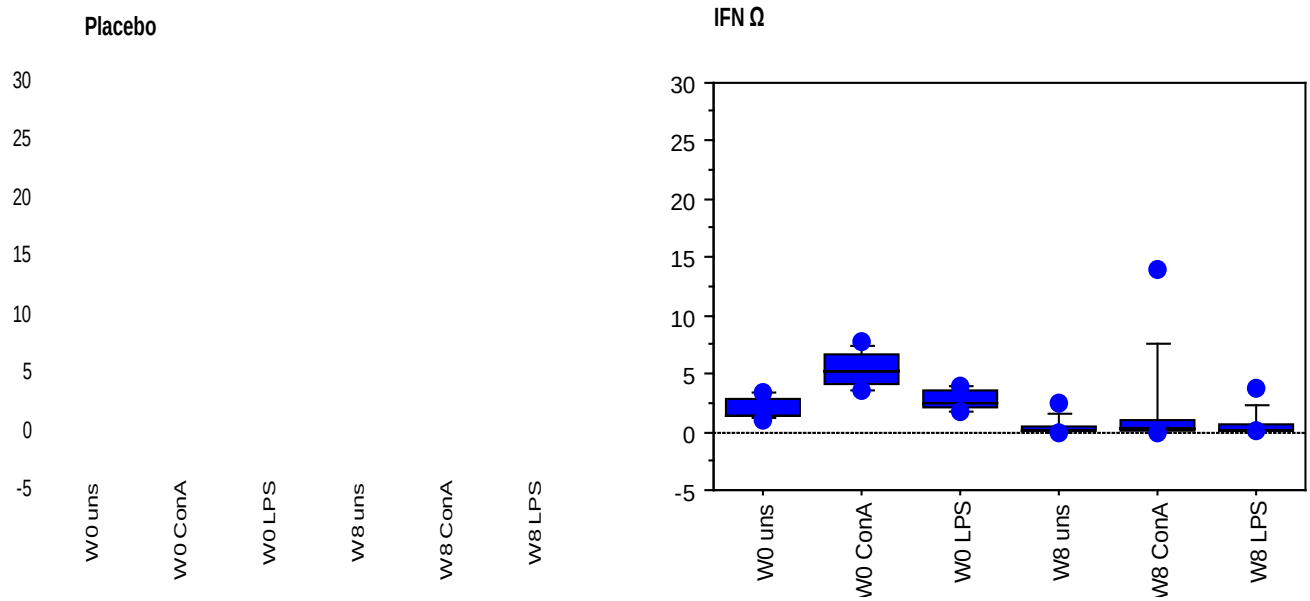
Interferon gamma mRNA could never be measured in any of the samples throughout the course of the observation period. Therefore, expression of interferon gamma was always below the detection limit of our assay.

4.4.6. Detection of GAPDH in stimulated PBMCs:

In order to detect differences in the cytokine response between the two groups, PBMCs were purified in week 0 and in week 8 from heparinised blood samples and stimulated under different conditions. In fig. 41 the results of GAPDH mRNA extracted from non-stimulated, con A stimulated and LPS stimulated lymphocytes in placebo and IFN Ω group performed in week 0 and in week 8 are displayed. GAPDH expression in unstimulated lymphocytes of placebo group was significantly higher than in IFN Ω group, although the differences were minimal. All other mRNA concentrations did not differ significantly.



Frequency distribution:



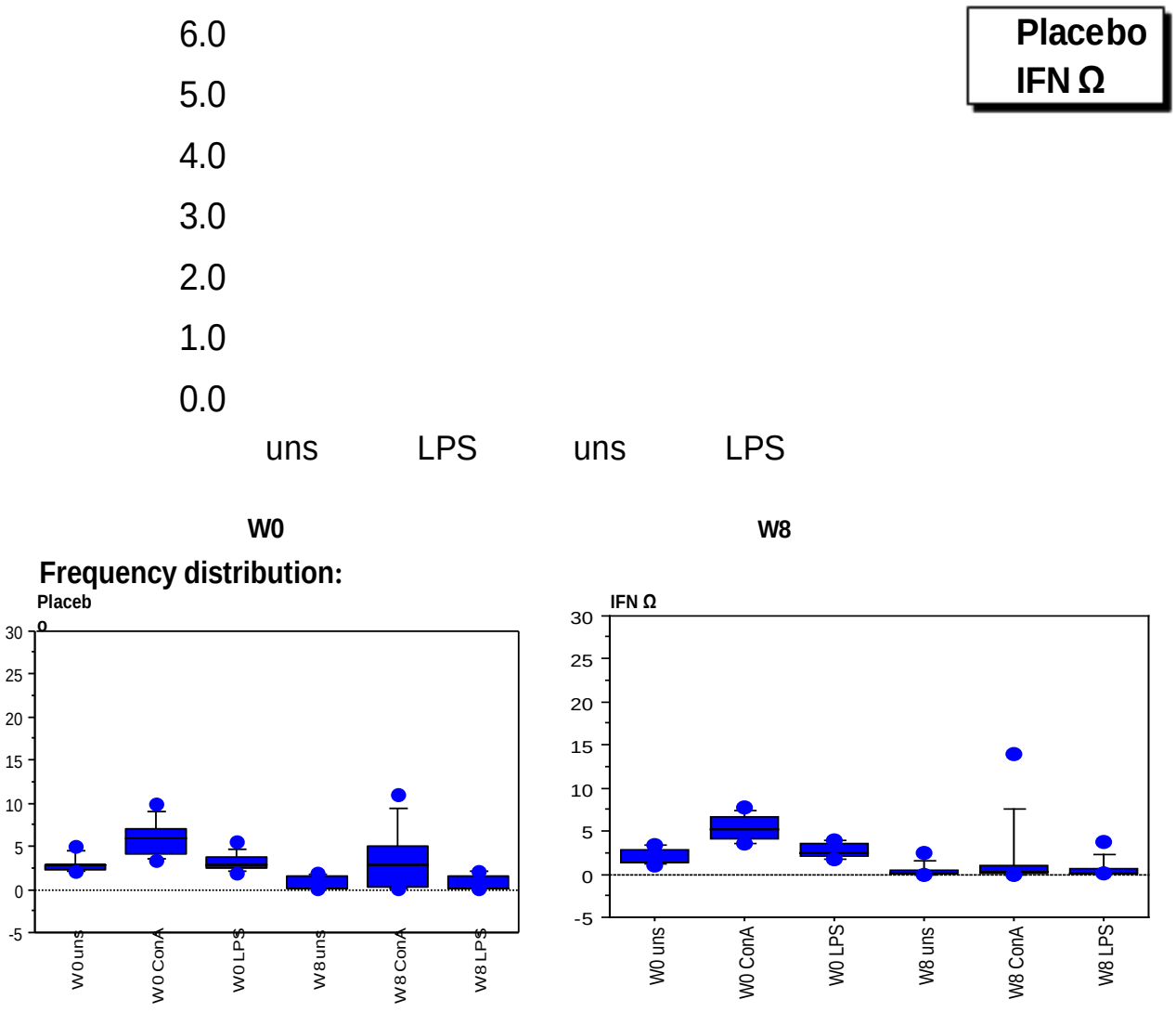
Significance between groups (p-values):

	W0				W8		
Groups	uns	ConA	LPS		uns	ConA	LPS
Placebo/ IFN Ω	0.0262						

Fig. 41: GAPDH concentrations in stimulated PBMCs

4.4.7. IL-10 to GAPDH ratio:

The IL-10: GAPDH ratio was measured in lymphocytes stimulated as mentioned in paragraph 4.4.6. (fig. 42). As before, the ratio of IL-10 to GAPDH was significantly different in the non-stimulated lymphocytes of week 0 while the IL-10 to GAPDH ratio in the con A and LPS stimulated cells did not differ significantly. In week 8, conA stimulated lymphocytes had a significantly higher IL-10: GAPDH ratio than the unstimulated and the LPS stimulated lymphocytes.



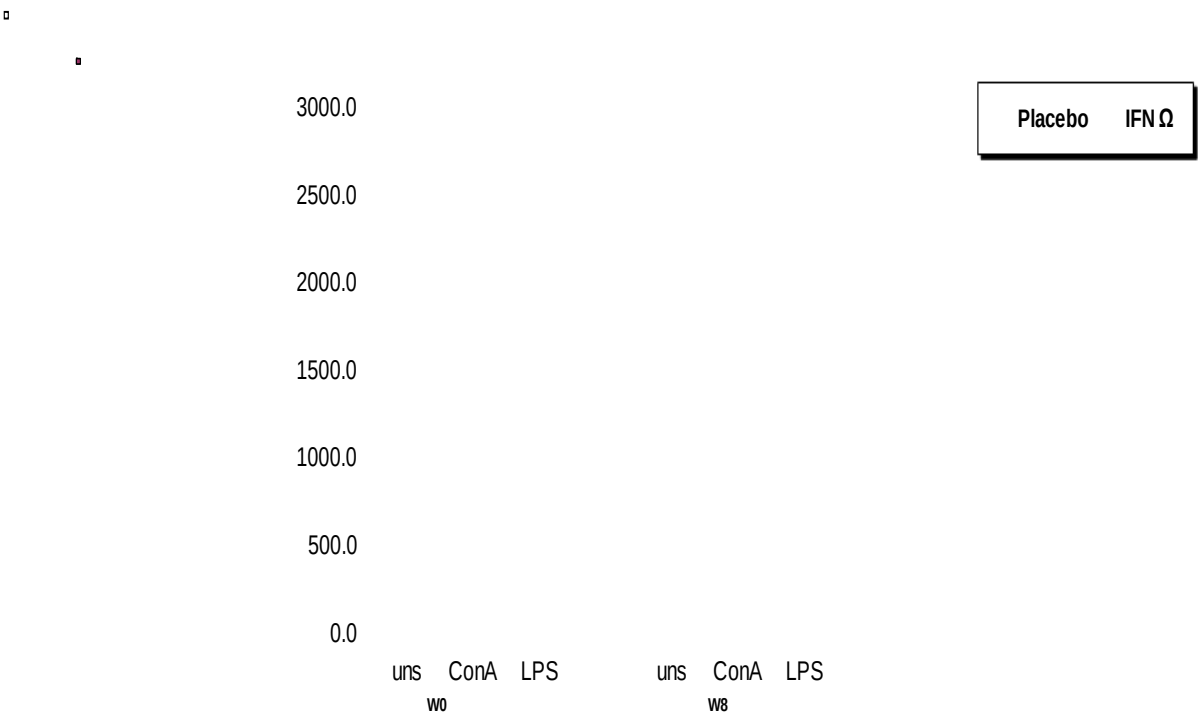
Significance between groups (p-values):

	W0				W8		
Groups	uns	ConA	LPS		uns	ConA	LPS
Placebo / IFN Ω						0.0019	

Fig. 42: IL-10: GAPDH ratio in stimulated PBMCs

4.4.8. IL-12 p40 to GAPDH ratio:

In week 0 and week 8 the ratio of IL-12 p40: GAPDH did not differ in any of the three stimulation conditions (fig. 43).



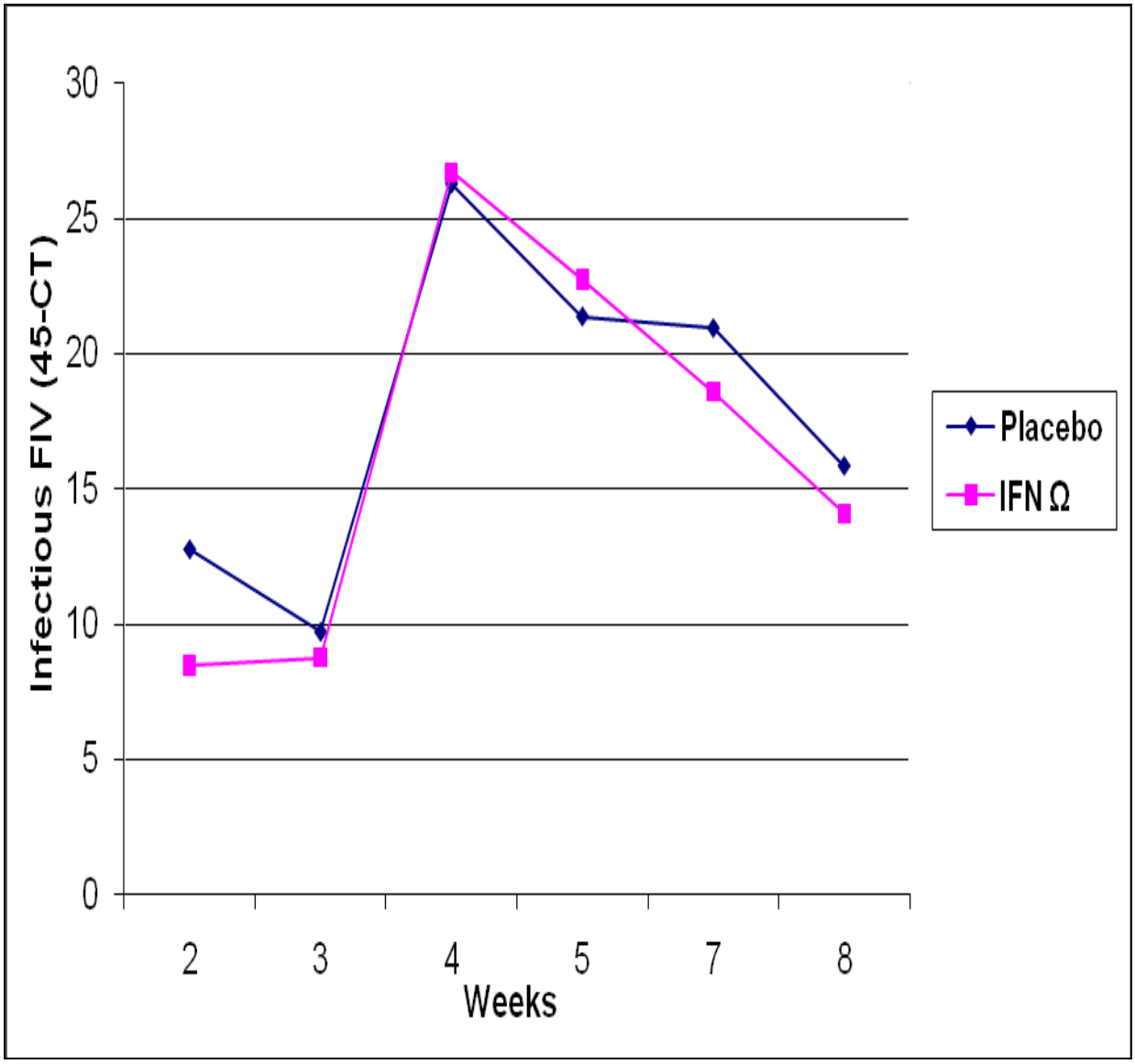
Significance between groups (p-values):

	W0			W8		
Groups	uns	ConA	LPS	uns	ConA	LPS
Placebo / IFN Ω	0.0680			0.0604		

Fig. 43: IL-12p40: GAPDH ratio in stimulated PBMCs

4.5. Virus isolation:

From all blood samples lymphocytes were purified and cultured for a duration of 4 weeks in order to isolate virus from the cats. Due to contaminations of the cultures results of week 0 and week 1 were not available. Cultures of week 2 till week 8 grew well and could be used for quantitation by real-time RT-PCR of isolated virus. The supernatant of these cultures showed identical levels throughout the course of the experiment (fig. 44). No significant differences between the two groups were found.



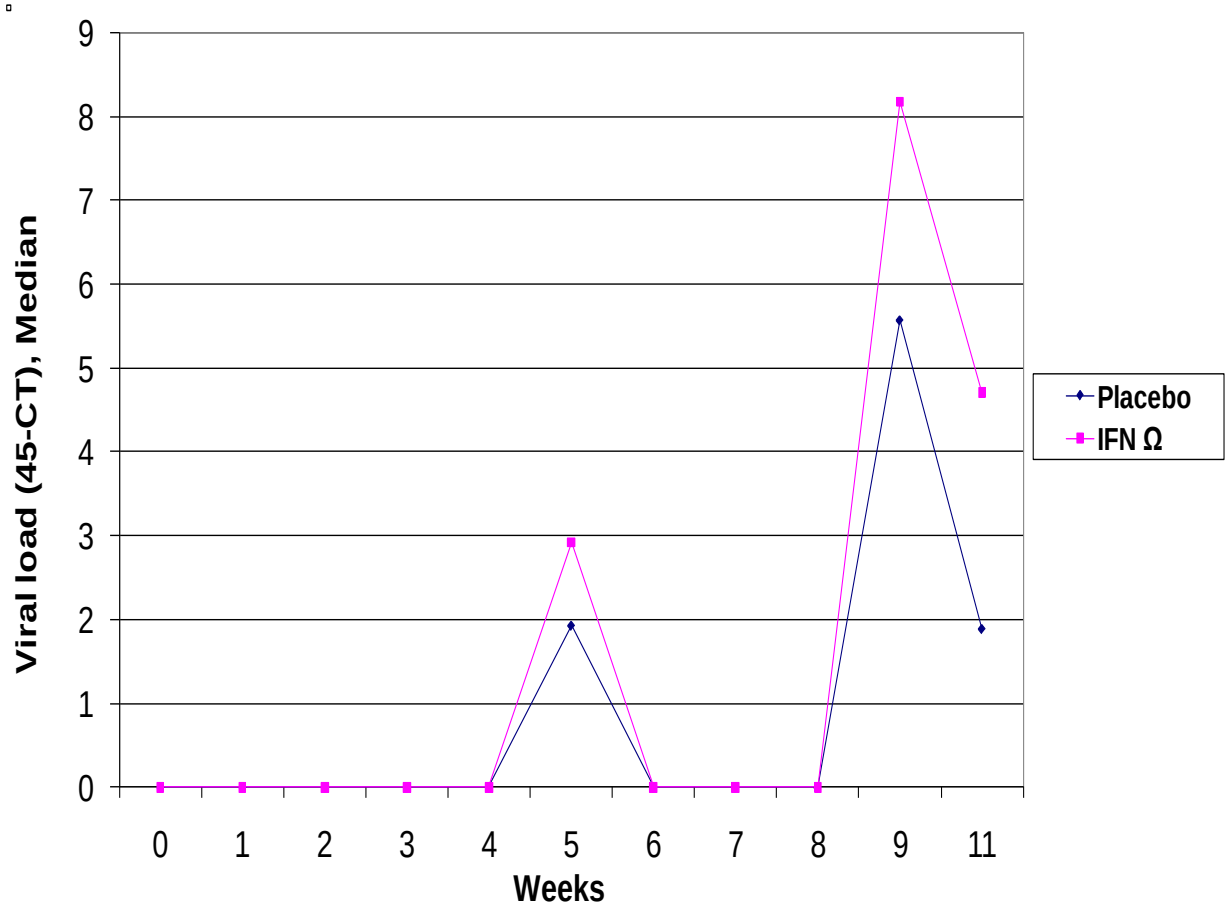
Significance between groups (p-values):

WEEKS	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
Placebo / IFN Ω													

Fig. 44: Virus isolation in culture during week 2 till week 8 of the experiment

4.6. Detection of FIV RNA in saliva:

Saliva samples were collected from all cats throughout the duration of the experiment. Swabs were used to extract RNA, which was then analysed for presence of FIV by real-time RT-PCR. The results are shown in fig. 45. It can be recognised that only in week 5 and week 9 and 11 FIV could be detected at relatively low concentrations. No significant differences were observed between the two groups.



Significance between groups (p-values):

	w0-5d	w0-3d	w0	w1	w2	w3	w4	w5	w6	w7	w8	w9	w11
weeks													
Placebo / IFN Ω													

Fig. 45: FIV RNA concentrations in saliva during the experiment

5. Discussion and conclusion:

5.1. Discussion:

5.1.1. General comments:

FIV is a lentivirus that infects members of the family Felidae worldwide {Burkhard and Dean, 2003}. As show in table 1 the prevalence of FIV infection is relatively low in Switzerland; the other countries have higher prevalences. Currently, no etiologic treatment of FIV infection is available that can be used under practical field conditions. One study demonstrates that the clinical course of FIV infection can be positively affected by treatment of infected cats with IFN Ω {de Mari et al, 2004}. So far, no information has been available on the prophylactic treatment of cats.

Yamamoto et al {2001} reported that IFN Ω has marked anti viral activity against FIV in vitro. It was the goal of this study to evaluate whether prophylactic treatment of cats with IFN Ω would lead to a decreased susceptibility against FIV. Unfortunately we did not observe significant effects of prophylactically administered IFN Ω against subsequent infection with low dose of FIV challenge.

If this had been the case, it would have been possible to treat cats prophylactically before they are brought into an environment with increased pressure of infection by FIV, such as catteries.

We expected that at least some of the cats in the treatment group would show resistance against FIV infection, delayed seroconversion or lower viral loads than the placebo-treated control group.

In the following sections, different aspects of the project are discussed.

5.1.2. Concept:

The aim of our project was to elucidate the influence of the feline Interferon Ω as prophylactic treatment of FIV. Twenty cats of the same age were divided into two groups and were kept under identical SPF conditions; food provided to the animals was identical. The two groups were free of the most important feline pathogens when they arrived at our facility and throughout the experiment (FeLV, FHV-1; FPV, FCoV and FCV). They were treated prophylactically by subcutaneous injection with either IFN Ω or placebo over a period of five days (1MU/kg daily).

Twenty four hours after the 5^{th} injection of IFN Ω or placebo all cats were injected intraperitoneally with 100 CID FIV GL_8 Virus which had been previously titrated in vivo and kindly donated by Prof. M. Hosie and Prof. O. Jarret from the University of Glasgow, Great Britain. The intraperitoneal challenge route was used because this procedure closely mimics the natural mode of transmission in cats, which occur mainly by biting {Pedersen,

et al. 1989}. All cats used in this experiment were negative for FIV infections on the day of challenge by virus inoculation.
The IFN Ω treatment was well tolerated by the cats, an observation which is in agreement with observations made by veterinarians in clinics and is also documented in the instruction for use of the product.

5.1.3. Detection of FIV Proviral load:

The FIV proviral load was determined weekly throughout the duration of the experiment using the real-time Taq Man assay developed by Leutenegger et al {1999}. The assay is able to detect as little as 1 FIV DNA copy per PCR. FIV proviral DNA was detected already 1 week after challenge infection in both, the IFN Ω and the placebo-treated cats. From the observation that no differences in proviral load were observed between the cats we concluded that the effect of IFN Ω must have been minimal or absent.

5.1.4. Detection of FIV viral load:

FIV RNA load is considered a parameter reflecting viral replication {Malmsten, et al. 2005} and was determined by the method decribed by Klein and colleagues {Klein, et al. 2001}. FIV RNA was detected in week 1 after challenge and reached a peak already in week 2 in both groups. The viral load ranged between 0.1×10^3 to 3.7×10^3 RNA copies / ml plasma with a mean of 1.9×10^3 copies / ml plasma in both groups. As the differences between the 2 groups were never significant, it was concluded that IFN Ω did not have a measurable effect on the FIV challenge infection. Therefore, measuring the FIV RNA load also supported the observation that IFN Ω had no prophylactic effect on FIV challenge infection. Several studies have shown that plasma viral RNA load increased shortly after infection to an initial peak in acute phase {Gomo, et al. 2002}. Plasma viral RNA load has been used as an indicator of the efficacy of antiviral therapy in HIV infection {O'Brien, et al 1996}.

5.1.5. Hematology:

The various red cell parameters observed in cats of the 2 groups differed significantly on several occasions (fig.5 – fig. 10). They also differed already in week 5 before challenge infection, i.e. even before begin of IFN Ω - treatment. These differences between the 2 groups can be explained by the fact that at the beginning of the experiment, the cats had to be assigned to the 2 groups by random criteria. However, also siblings had to be distributed evenly to the 2 groups in order to minimize genetic influence. In this context some animals assigned to the placebo group by coincidence had a higher body weight (fig. 3) which was maintained throughout the entire observation period. The higher body weight

was correlated with higher values of red cell counts (fig. 5), higher hematocrit (fig. 6), higher hemoglobin values (fig. 7), lower MCV, lower MCH, lower MCHC (fig. 8-10). Therefore, the differences in red cell parameters between the 2 groups cannot be assigned to the effect of the IFN Ω - treatment. Absence of significant difference for the parameters platelets, total leucocyte counts and neutrophil counts (fig. 11, 13 & 14) also confirms absence of significant effects induced by IFN Ω. However, in week 3 after challenge infection, the lymphocyte counts of the IFN Ω - treated cats were significantly higher than those of the placebo group. In addition, the lymphocyte counts of the IFN-ω-treated cats had tendency to be higher than those of the placebo group during the first 4 weeks. As FIV is known to induce a transient drop of lymphocyte counts during the early (and late) phase of the infection {Leutenegger, et al. 1999} this may reflect an effect of IFN Ω - treatment. Similarly, the increased values of monocytes in the IFN Ω - treated cats between weeks 3 and 9 after infection (fig. 16) could also be considered indicative of a low-level IFN Ω -effect, the lower eosinophil counts in the IFN Ω - treated cats at the end of the treatment (fig. 17) also support the concept that IFN Ω - treatment must have had an effect albeit minor. The basophil counts (fig. 18) were lower in the IFN Ω - treated cats at the end of treatment, an effect that may also be attributed to IFN- Ω - treatment.

5.1.6. Clinical chemistry:

The parameters ALAT, ASAT, AP, lipase, bilirubin, glucose, urea, creatinin, protein, albumin, cholesterol, sodium, potassium, chloride, calcium and phosphate were measured weekly and no significant differences between the two groups were observed during the entire observation period. From these results it was concluded that IFN Ω - treatment had no detectable effect on these parameters. For the parameter glucose, a sharp drop was observed in week 6 (fig. 27), this can be explained by the fact that on the day of collection, blood samples were collected later than usual. As the cats were not fed prior collection in order to avoid hyperlibidemia, the glucose concentration had decreased. Obviously, this drop had nothing to do with FIV infection and IFN Ω - treatment. A significant decrease in the urea values in week 5 in the cats of the placebo group cannot readily be explained. It could be possible that on the day before blood collection the cats of the placebo group which were housed in a separate room were fed several hours earlier than those of the IFN Ω - treated group.

A drop in the concentration of sodium and chloride 1 week after challenge could be related to FIV infection. To our knowledge, such changes in sodium and chloride concentrations have not been documented before.

5.1.7. Serology:

The levels of FIV-specific antibodies were detected by ELISA using recombinant transmembrane antigen {Calzolari, et al. 1995}. The antibody increased at week 3 following the challenge infection and increased to the peak at week 5, then decreased afterwards (fig. 21). However, no significant differences were observed between the two groups during the observation period except at week 8.

5.1.8. Cytokines:

Cytokine expression is significantly affected by FIV infection in the early phase of infection (months to years) when the viral load is kept low by the cats immune system. Cytokines typical for the Th1 response predominate. These are essentially IFN Ω and IL-12 which increase during early infection while the typical Th2 cytokines IL-4, IL-5, IL-6 and also IL-10 increase later {Burkhard, et al. 2003}.

For our study, we decided to monitor prospectively the expression of IL-10 and IL-12. It would have been desirable that the cytokines of interest could be measured at the protein level. However, ELISAs or other immunological assays for feline cytokines do not exist and would be difficult to develop. Therefore, we decided to study the expression on the mRNA level and to relate the specific cytokine expression to the expression of a housekeeping gene, in our study GAPDH {Leutenegger, et al. 1999}. The ratio of cytokine mRNA to GAPDH mRNA allows for normalization for cell count and expression as housekeeping genes are thought to be expressed at a constant rate {Leutenegger, et al. 1999}. In the present study, we determined cytokine expression in blood lymphocyte ex vivo and in addition in PBMCs stimulated by concanavalin A or LPS.

5.1.8.1. In blood:

The expression of GAPDH was relatively constant over the entire observation period. The values were significantly lower for the IFN Ω - treatment group during the treatment period before challenge (fig 38). This lower expression may be related to the IFN Ω - treatment during the first 7 weeks of the FIV infection. In contrast, GAPDH- expression had a tendency to be higher in the IFN Ω - treated cats. Again, this may reflect an effect mediated by the IFN Ω - treatment. The ratio of IL-10 to GAPDH-expression changed enourmously during FIV challenge infection; however, there was no difference between the 2 groups. In contrast, the ratio of IL-12 p40 to GAPDH showed almost no changes over the time course of FIV-infection and, here too, no differences between the 2 groups were observed. In previous experiments with experimental FIV infection, we observed a strong IL-12 expression already a few weeks after infection {Boretti, et al. 2000}. Absence of this IL-12 increase could be explained by the FIV strain Glasgow 8 which was used. In previous experiments, we had used the strain Zurich2 {Leutenegger, et al. 2000}.

IFN Ω mRNA could be detected throughout the observation period which indicates that the cats of both groups did not develop a strong Th1 response during infection with FIV Glasgow 8.

5.1.8.2. In stimulated PBMCs:

From the fact that no difference in GAPDH expression in stimulated PBMCs was observed, it was concluded that treatment with IFN Ω did not have an effect on PBMCs, neither in week 0 nor in week 8. Interestingly, there were significant differences in the ratio of IL-10 to GAPDH expression on 2 occasions: in week 0 for the unstimulated and in week 8 for the Con A-stimulated PBMCs. While the difference in week 0 can be attributed to the IFN Ω - treatment, it is difficult to imagine how the difference in week 8 for the Con A-stimulated cells can be explained. As with IL-10, also the IL-12: GAPDH ratio was almost significant in week 0 in unstimulated PBMCs. This too can be considered an effect of IFN Ω - treatment.

5.1.9. Virus isolation in cell culture:

The two groups showed identical levels of FIV isolated in culture during the observation period. The virus grew well and the peak was at week 4 post infection (Ct = 19). From this it was concluded, too, that IFN Ω - treatment did not affect the course of FIV infection.

5.2. Conclusion:

Treatment by IFN Ω in vivo was found not to have a detectable effect on virus load, provirus load and infectivity. Minor effects were found with respect to hematological parameters and cytokines. Therefore, the main goal that cats can be non-specifically protected against low dose FIV infection was not reached. Thus, prophylactic FN Ω - treatment cannot be recommended to protect even partially against FIV infection in high risk environment.

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